

POLYANION – PLASMA PROTEIN INTERACTIONS

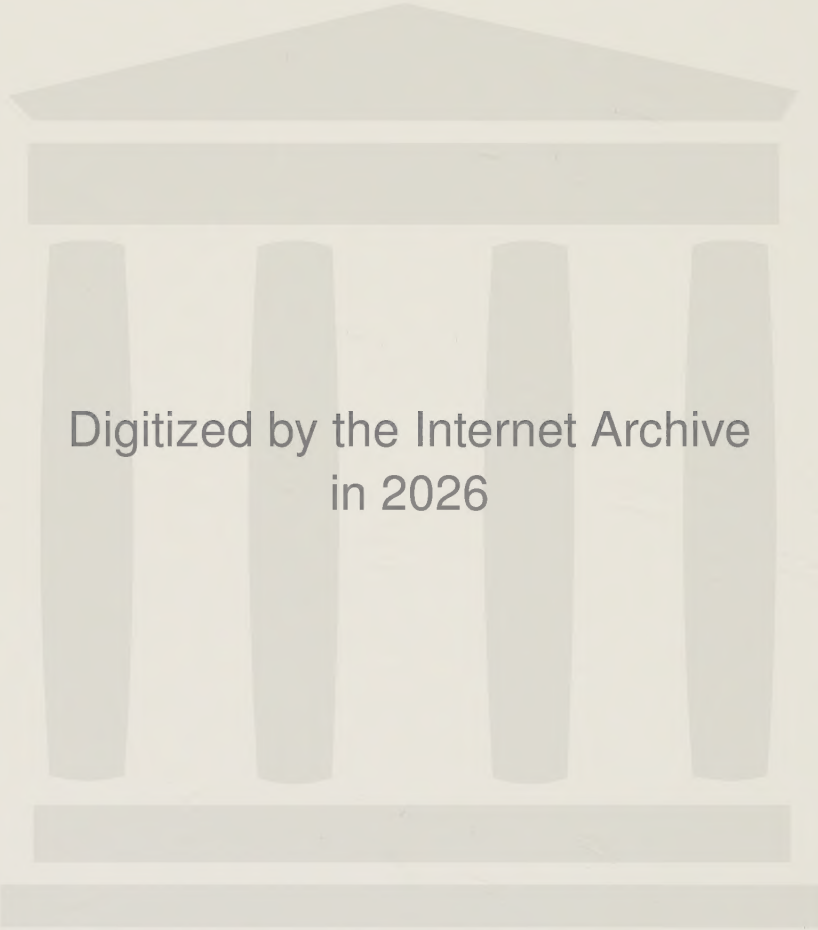
Studies concerning heparin binding with antithrombin
and the first component of complement
agarose-protein binding

E. John McKay



Malmö 1981

Lund 1981



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POLYANION - PLASMA PROTEIN INTERACTIONS
STUDIES CONCERNING HEPARIN BINDING WITH ANTITHROMBIN
AND THE FIRST COMPONENT OF COMPLEMENT
AGAROSE - PROTEIN BINDING

E. JOHN MCKAY

MALMÖ 1981

This dissertation is based on the following articles:

- I Immunochemical analysis of active and inactive antithrombin III.
E.J. McKay. Brit. J. Haematol. (1980) 46, 277-285.
- II The interaction of heparin with plasma proteins. Demonstration of different binding sites for antithrombin III complexes and antithrombin III. E.J. McKay and C.-B. Laurell. J. Lab. Clin. Med. (1980) 95, 69-80.
- III A simple two-step procedure for the isolation of antithrombin III from biological fluids. E. John McKay. Thromb. Res. (1981) 21, 375-382.
- IV Practical requirements for immunochemical analysis of antithrombin III in agarose gels. E. John McKay. Clin. Chim. Acta (1981) (in press).
- V Activation of C1, the first component of complement, the generation of C1r-C1s and C1-inactivator complexes in normal serum, by heparin affinity chromatography. E. John McKay, Anna-Brita Laurell, Ulla Mårtensson and Anders G. Sjöholm. Molecular Immunology (1981) 18, 349-357.
- VI Binding of purified C1 subcomponents, C1 inactivator and their complexes to immobilized heparin. E. John McKay, Ulf Johnson, Anna-Brita Laurell, Ulla Mårtensson and Anders G. Sjöholm. Acta Path. Microbiol. Scand., Sect. C (1981) (in press).
- VII A simple two-step procedure for the purification of plasma C1q from different animal species. E. John McKay. Immunology Letters (1981) (in press).

The above articles will be referred to in the text according to their Roman numerals.



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INTRODUCTION

Naturally occurring sulphated polysaccharides are linear polyanions most of which have colloidal characteristics and possess a remarkable structural uniformity (1,2). These high molecular weight anionic polymers also possess rheological and complexing properties that have stirred the interests of biologists for some time. A rich source of these naturally occurring sugars are found in seaweeds (carrageenans, agar, agarose sulphate) where they probably serve structural functions and act as natural absorbants to hold large volumes of water for proper functioning of the sea plant (3). In mammalian tissues they serve similar roles, but in addition provide emolliency and lubrication (4,5). The best known mammalian glycosaminoglycans are heparin, heparan sulphate, dermatan sulphate, hyaluronic acid and chondroitin sulphates. Simpler sulphated polyanions have been synthesized and are commercially available (cellulose sulphate, dextran sulphate and sodium pentosan polysulphate).

Glycosaminoglycans are linear polysaccharides consisting of alternating uronic acid residues that are usually in covalent association with protein (proteoglycans) (6). Electron microscopy data implies that proteoglycans have features similar to a double edged comb with the polysaccharide chains extending at right angles from a central protein core (7,8). Glycosaminoglycans can be distinguished by their differing structural configurations, induced by charge group variation and/or interchain binding, in addition to the amount and location of sulphate groups (9). It is probably these subtle structural differences that produce dramatic differences in their biological properties (10). Such an example is the marked difference in anticoagulant behaviour between heparin and heparan sulphate. Glycosaminoglycans interact with many proteins, e.g. in the structural organization of the extracellular matrix (heparan sulphate and fibronectin) (11), the control of haemostasis

(heparin and antithrombin) (12), specific binding of plasma proteins to blood vessel walls (heparan sulphate and lipoproteins) (13), and the regulation of cell behaviour (heparin and chondroitin sulphates located in the basophilic granules of mast cells) (14).

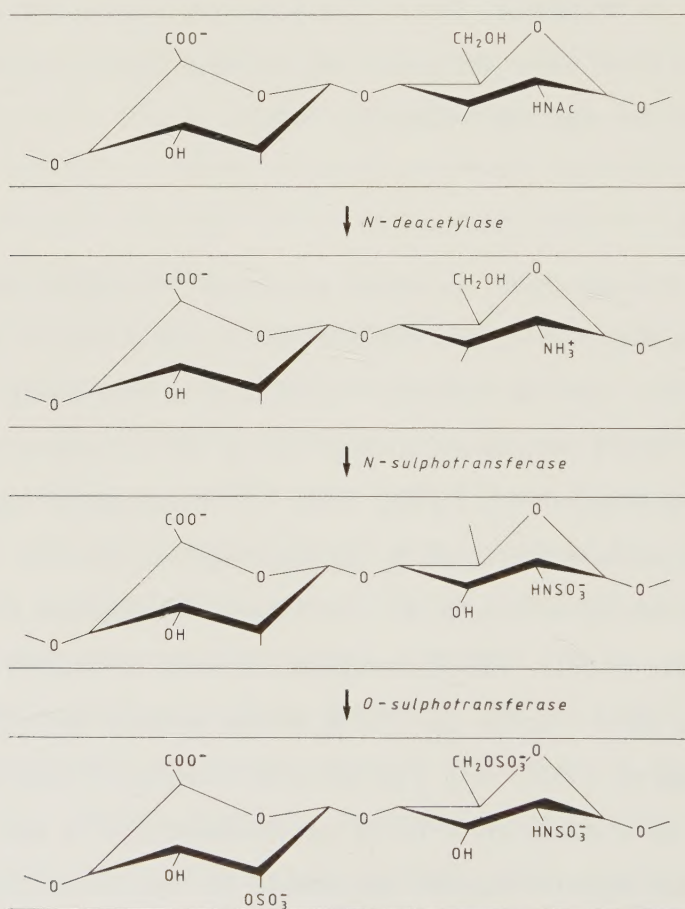
These studies are primarily concerned with the interaction of anti-thrombin and complement with two of the better known polyanions - heparin and agarose sulphate. *Heparin* is probably best known for its anticoagulant activity, yet despite being used as such for the last 50 years, elucidation of structure and mode of action is still incomplete. *Agarose* has attained importance in the biomedical field because its physicochemical properties approach those of an ideal gel matrix and has been used extensively as a support medium for zone electrophoresis (15).

Heparin

Heparin is a straight chain, sulphated polysaccharide polymer that consists of alternating uronic acids and glucosamines to form a twofold helical structure (9,16). Less well ordered regions may be interspersed between the better defined block structures, giving rise to the heterogeneity observed in most heparin preparations (10,16) (Fig. 1). Polydisperse heparin, however, has considerable variation in its anticoagulant activity that has been related to its source of origin (9), its chemical composition (17), and its molecular weight (9,16). The anticoagulant activity of heparin would appear to reside in a small fraction pertaining to one third of unfractionated commercial heparins (18). This high activity fraction of heparin is characterized by a strong affinity for antithrombin III. A detailed structure of the heparin sequence responsible for the strong interaction has recently been reported (19). This sequence was obtained by isolating a

heparin fragment which remained bound to immobilized antithrombin after degradation with a bacterial heparinase (19). This fragment proved to be 12-16 sugar residues long containing a non-sulphated L-uronic acid (20).

Although the ultimate correlation between structure and function of heparin still remains elusive, it is evident that its anticoagulant activity is related to an extremely small fraction of total heparin with precise structural features in the monosaccharide sequence. However, the helical order of heparin may also contribute to the formation of specific binding sites.

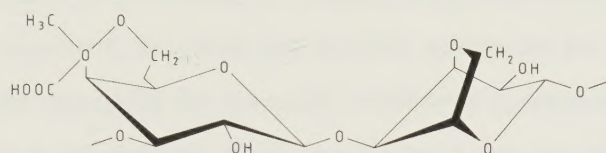


A proposed sequence of polymer-modification leading to formation of predominant disaccharide unit of heparin

Agarose

Agarose is the principle gelling component in agar which can be separated from other highly charged fractions collectively known as agaropectin (15, 21).

Agarose is a linear carbohydrate polymer of 1,3-linked β -D-galactopyranose and 1,4-linked 3,6 anhydro α -L-galactopyranose residue. Both agarose and agaropectin have the same backbone structure but the latter has varying amounts of sulphate esters, pyruvic acid and methyl groups (21). (See Fig. 2.)



Agarobiose subunit of agarose showing position of pyruvate ketal

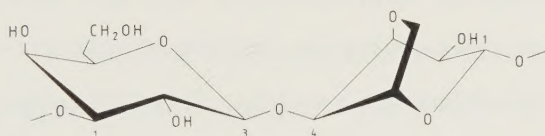
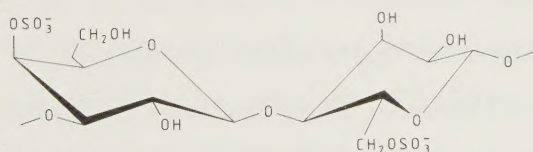


Fig. 2

Agarobiose: Basic repeating unit of agarose



Agarobiose subunit of agarose showing position of sulphate

Most of the information regarding the three-dimensional structure of the agarose gel comes from X-ray studies combined with data for changes in optical rotation during the sol-gel transition (22,23). These results support a parallel left-handed double helical structure. Water molecules contained within the central cavity participate in hydrogen bonding between the strands and contribute to its stabilization (23). Sulphate groups apparently protrude outwardly from the carbohydrate backbone and do not contribute significantly to the stabilization of the gel network (23). These "extra" sulphate groups provide freely available charged surfaces (in the form of anionic binding sites) throughout the agarose matrix to which certain proteins and/or cations can directly interact.

The gel can contain more than 100 parts of water for each part of agarose (23), leaving relatively large voids through which macromolecules and bioparticles can pass freely. Gels of 1 % (w/v) agarose apparently have exclusion limits for proteins of $\approx 10^8$ daltons, and thus allow for the development, diffusion and electromigration of antigen-antibody complexes without consequent sieving effects.

For most agars a clean separation between agarose and agarpectin can not be achieved and therefore the resulting polysaccharide ought to be regarded as a spectrum of continuously varying covalent structures with agarose being an idealised extreme (21,23). Consequently agarose, by generally accepted convention, contains less than 0.7 % sulphate (24).

Polyanion: plasma protein interaction

Interaction between polyanions and plasma proteins can be divided into two categories: one non-selective, the other highly selective. Non-selective binding is primarily due to the high charge density of the carbohydrate chains (25). Examples of plasma proteins that bind to polyanions non-

-selectively include lipoproteins (26), lipoprotein lipase (27), fibronectin (28) and Clq (29). Stereospecific factors have also been recognised (25), e.g. heparin and heparan sulphate bind some proteins with higher affinity than do chondroitin sulphates (10,25), even though charge densities are similar. This has been attributed to the L-iduronic acid in the former polyanions (10). That binding of polyanions to plasma proteins can invoke conformational changes in the said proteins (26) may contribute to these stereospecific factors.

In certain instances a more selective binding occurs that cannot be substituted by another polyanion, e.g. glycosaminoglycans other than heparin do not bind antithrombin (27). Presumably this is related to a specific sequence in heparin molecules not present in other glycosaminoglycans (20). However, agarose sulphate and carrageenans can bind antithrombin although less tightly than heparin (21).

The interaction between sulphated polyanions and certain proteins can induce precipitation, e.g. lipoproteins and Clq (26,29). Low density lipoproteins form insoluble complexes with heparin or dextran sulphate in the absence of cations, although the latter facilitates precipitation. High density lipoproteins, in contrast, require high concentrations of both cations and polyanions for precipitation (29,30).

Heparin-antithrombin interaction

Heparin requires a cofactor in plasma for optimal anticoagulant activity which has been identified as antithrombin, a protease inhibitor with a molecular weight of 65,000 (31,32). Heparin accelerates the progressive rate of inhibition exerted by antithrombin on thrombin (33), Factor IXa (33), Factor Xa (33), Factor XIIa (33) and kallekrein (34). Irreversible mole:mole covalently bound complexes between the enzymes and inhibitor are

normally formed, and heparin accelerates their rate of formation without altering the stoichiometry (33). Rosenberg and Damus (33,35) demonstrated that the lysine groups in antithrombin serve as binding sites for heparin and that arginine residues are involved in the irreversible complex formation with the active centre serine of the enzyme. Heparin apparently induces a conformational change in antithrombin on binding so that the arginine binding sites become more accessible. It would appear that heparin acts in a catalytic fashion (33,36) as one molecule of heparin can promote the binding action of several antithrombin molecules to thrombin. In addition antithrombin-protease complexes have lower affinity than non-complexed antithrombin for heparin (37). Therefore, in light of current knowledge, heparin should perhaps be regarded as the cofactor for antithrombin rather than the reverse. The importance of antithrombin in the coagulation system is illustrated by the increased susceptibility to thrombosis in persons with depressed antithrombin levels (38,39).

The complement system

The complement system comprises some twenty individual plasma proteins, many of which circulate as zymogenic proteins (40). Activation triggers off a sequential, cascade-like series of events that leads to irreversible alterations of cell membranes and cell death (40). Other effects are produced during the activation phase, mediated by different components within the system, e.g. kinin activation (41), immune adherence (42), chemotaxis (42) or histamine release from mast cells (43). Constraints within the system are made by spontaneous decay of the activated products and by several regulating or control proteins that operate at specified points during the activation and assembly phases. Control proteins, C1 inactivator, C3b inactivator, β IH, and C4 binding protein have been defined.

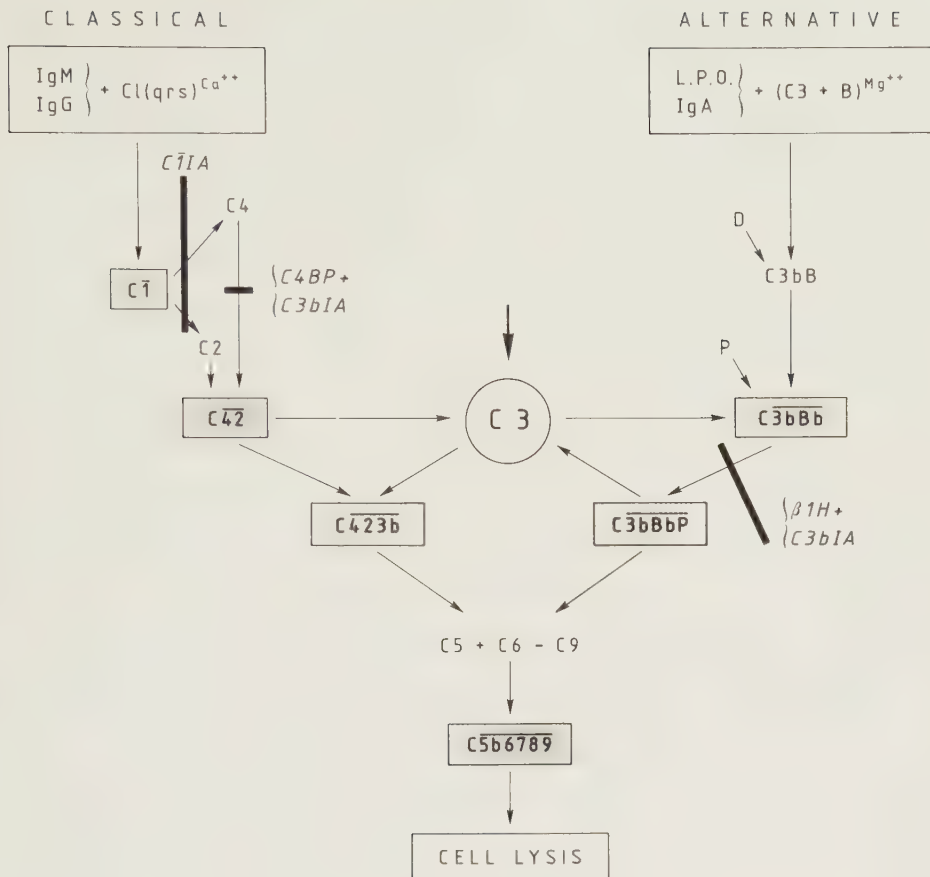


Fig. 3. Schematic diagram of the complement cascade*. Hatched areas denote activated macromolecular complexes. Solid lines denote points of inhibition.

*The nomenclature used is that recommended following discussions at several international conferences (44,45). The components of the classical pathway are denoted with the letter C and numbered, C1-C9. Capital letters are used for the alternative pathway components. P stands for Properdin, B for factor B (previously known as C3 proactivator, C3PA, or glycine rich β -glycoprotein, GBG. D denotes factor D (previously known as precursor of C3PAase or pro-GBGase). Enzyme activity of individual or complexed components is indicated by a bar over the component number, eg. C1s, C42. The nature of fragments arising from cleavage or activation is denoted by a small letter, eg. C3a, C3bBb.

Activation of C3 can be induced by two distinct mechanisms - the classical and alternative pathways (Fig. 3). The classical pathway of activation is mediated by immune complexes containing IgG or IgM (46), that activate the first component of complement (C1), a macromolecular complex held together by calcium comprised of subcomponents C1q, C1r and C1s in the molecular ratio of 1:2:2 (40). Subcomponent C1q binds to the Fc portion of IgG or IgM thereby inducing internal activation of C1r. The only known natural substrate of C1r is C1s that is converted into an active protease $\overline{C1s}$ by cleavage of a single peptide bond (47). Activated $\overline{C1r}$ and $\overline{C1s}$ are inhibited by $\overline{C1}$ inactivator (48). Enzymic $\overline{C1s}$ cleaves C4 and C2 into C4a and C4b and C2a and C2b respectively. Fragment C4b can bind covalently to cell membranes and immune complexes (49). In the presence of magnesium, C4b forms a complex with C2 generating the classical pathway C3 convertase, $\overline{C42}$ (49).

The alternative pathway can be activated directly by complex polysaccharides contained in yeast or bacterial cell walls and provide immediate defence against infections before the development of immunity (50). Generation of this pathway requires C3b, factor B, factor D and magnesium. Fragment C3b forms a magnesium dependent complex with B (51), that is activated by D, cleaving B in the complex to release Ba fragment (51). The resulting complex, $\overline{C3bBb}$, i.e. the alternative pathway C3 convertase, can induce a positive feedback mechanism cleaving C3 to recruit more C3b for its own perpetuation (the so-called amplification loop).

Both forms of C3 convertase modulate the C3b substrate specificity to enable it to cleave C5. The formation of C5b6789 appears to be a self-assembling process which leads to part of this macromolecular complex being inserted into the cell membrane inducing the lytic phase (52).

Polyanion-complement interaction

Heparin has long been known to possess anticomplement effects (53). Previous studies have identified many possible sites of inhibition by heparin ranging from the early activation phases to the later assembling units.

Heparin appears to interact with C1 in several ways. Raeppe et al. (54) demonstrated that heparin and other sulphated polyanions could inhibit C1q from binding to antigen-antibody aggregates. The cleavage activity of C1s on C4 and C2 could be prevented by the same sulphated polyanions, and inhibit the binding of C2 to C4b (54,55). However, there was no evidence for direct heparin interaction with isolated C1s (55). Indirect evidence suggested that heparin potentiated C1 inactivator inhibition of the esterolytic activity of C1s (56,57).

Activation of C1 by polyanions has been claimed by Cooper (58), and demonstrated recently by Burger et al. (59) using sulphated dextrans. Conversely, Strunk and Colten (60) were unable to show either activation of C1 or enhancement of C1-C1 inactivator complex formation in the presence of heparin. It has also been reported that heparin inhibits the assembly of the trimolecular complex C5b67 as measured by reactive cell lysis (61), and may modulate the amplification of C3 activation by direct interaction with specified proteins of the alternative pathway (62).

THE PRESENT INVESTIGATION

This investigation is concerned with the interaction of polyanions with certain plasma proteins. Particular emphasis has been placed on the direct binding of antithrombin (I, II, III, IV) and the first component of complement (V, VI, VII) to immobilized heparin. The effects of agarose-protein interactions on immunoprecipitation have also been considered (I, IV, VII). These interactions were studied by a variety of techniques, but in particular by combining the selectivity of affinity chromatography with specific immunochemical assays.

An essential requirement of affinity chromatography is that the ligand exhibits highly selective binding. This procedure can therefore be used both for isolation and identification of individual components that bind to the ligand and to characterize different binding processes between relatively complex partners, such as the differences in heparin binding between native and inactive antithrombin (II) or between zymogen and activated CIs (VI). Non-specific interactions may occur between proteins and the matrix backbone, the spacer arm between matrix and ligand, or the ligand (63). These unwanted interactions may be reduced or circumvented by manipulation of experimental conditions. These types of problems were encountered during the purification of active antithrombin (III) and CI_q (VII).

The specificity of antigen-antibody reactions make immunological procedures, such as electroimmunoassay, useful for identifying proteins eluted from the affinity columns. The high resolution of such methods coupled with the fact that results may be visually assessed are added advantages.

The interaction of two or more components to form a complex results in molecular weight differences, and often altered chromatographic or electrophoretic mobility. Electroimmunochemical methods are ideally suited to study such complexes provided one of the reactants is antigenically detectable and

changes in surface charge and/or precipitin morphology can be recognised. In this investigation such reactions were explored using 'specific' antisera directed towards the inhibitor protein-antithrombin or towards the serine proteases-C1r and C1s.

Ligands other than proteins may affect antigenic reactivity and this problem is considered where heparin and other sulphated polysaccharides bind directly to antithrombin (I, IV). Antibodies against serine proteases may demonstrate loss of antigenic reactivity when enzyme is in complex with its inhibitor protein as in the case of C1r in complex with C1̄ inactivator (V, VI) (64). Such reactions can be used to advantage in determining the activity status of proteins. However, the potential loss of antigenic reactivity, because of complex formation, in one or both of the reactants make quantitative analysis difficult. The functional activity of the protein is lost and the immunochemically determined quantity may bear little relationship to the amount of functionally intact protein. This problem is further complicated when different molecular forms of the same protein are present in the test sample. These problems are considered particularly in the section on antithrombin analysis and distinctions made between active and inactive protein (I-IV).

HEPARIN-ANTITHROMBIN INTERACTION

An antiserum that distinguishes active antithrombin from inactive antithrombin proteases (I, II, III)

Antibody responses are directed against antigenic determinants dictated by the three-dimensional structures conferred on proteins by their primary structure and unique folding of the peptide chains (65). Consequently altered structural conformations of the protein will be reflected by differences in antigenic reactivity, i.e. its capacity to interact with antibody (66). Also subtle changes associated with some forms of ligand binding may be sufficient to alter antigenic reactivity (67).

The high specificity of antigen-antibody interactions enables the entrapment of selected molecular forms of immune-complexes within the gel matrix (68). This relationship between different forms of a protein is usually determined using immunodiffusion tests, quantitative precipitin reactions or microcomplement fixation methods (69).

Immunodiffusion tests are somewhat insensitive in illustrating minor antigenic changes (69). Differences in intensity of the developed precipitin lines may reflect differences in affinity of the antiserum for its reactants, but absolute antigenic differences are not detected (70). Brager and Wilson (71) have demonstrated that quantitative precipitin tests are useful for illustrating small differences in antigenic reactivity between proteins that give lines of complete identity by immunodiffusion techniques.

The high resolving power of electroimmunochemical systems allows a clear distinction of proteins that are antigenically dissimilar and also the detection of different antigenic determinants on the same protein molecule which give rise to immunoprecipitation patterns of non-identity (72,73,74). Electroimmunochemical methods were employed to elucidate differences in precipitin patterns observed when two antisera ostensibly monospecific for

antithrombin were used.

Both antisera were initially characterized by double immunodiffusion (75). Plasma and serum, purified antithrombin, antithrombin-proteases (isolated from serum) and antithrombin-thrombin complexes yielded reactions of complete identity with both antisera. Cross-reactivity against purified prothrombin and/or thrombin in concentrations up to 0.5 mg/ml was not demonstrated. The intensity of the precipitin lines obtained with serum, antithrombin-protease complexes and antithrombin-thrombin complexes were noticeably greater with antiserum No 2 than with antiserum No 1 but no spurring was observed.

Antiserum No 1 (Fig. 4 A) demonstrated little variation in height or morphological appearance of the electroimmunoassay precipitin loops produced by identical blood samples taken into heparin, EDTA or as serum. In contrast, antiserum No 2 (Fig. 4 B) demonstrated considerable variation in both height and morphological appearance of the precipitin loops. In particular the serum sample demonstrated an additional dense precipitin loop inside a weaker, more diffuse precipitin loop. Heparin samples produced precipitin loop heights 25 % greater than EDTA samples that were in turn 30 % higher than serum. Further analyses of these same samples were made by electroimmunoassay but in the presence of calcium to induce clotting during electrophoresis (Fig. 4 C). The modification caused multiple precipitin arcs to be formed in both EDTA and serum samples whereas the heparin sample demonstrated diffuse inner trailing within a single precipitin loop. The addition of thrombin to purified antithrombin induced the formation of an additional dense inner precipitin loop with a corresponding decrease in the height of the outer more diffuse precipitin loop similar to that observed with serum. These findings implied that antiserum No 2 was capable of reacting with additional antigenic determinants on antithrombin that arose during the clotting process.

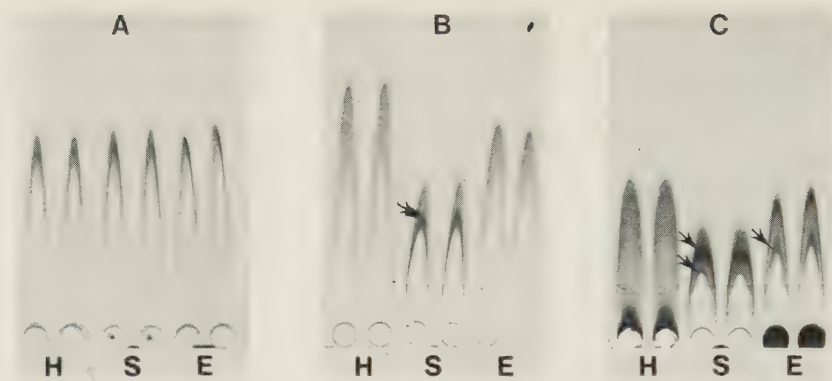


Fig. 4. Electroimmunoassay

- A) Antiserum No 1 - Identical samples taken as heparin, serum or EDTA.
- B) Antiserum No 2 - Arrow denotes additional precipitin loop recognising antithrombin-protease complexes in serum.
- C) Antiserum No 2 - As for B but run in presence of Ca^{2+} .

In order to confirm this interpretation further analysis was undertaken using crossed immunoelectrophoresis with serum and antithrombin-thrombin complexes. A previously reported modification (76) was used that involved the incorporation of heparin into agarose during electrophoresis in the first dimension. This procedure separates antithrombin from its protease complexes according to their different heparin affinities (76).

Figure 5 A illustrates the results obtained with antiserum No 1 using normal serum as the antigen source. The slower migrating α_2 antithrombin-protease complexes demonstrated a single precipitin arc which displays immunological identity with the faster migrating free antithrombin. However, using antiserum No 2, under the same conditions, an additional dense inner precipitin arc was produced with the antithrombin-protease complexes. Figure 5 C and D demonstrates similar reactions to B after thrombin has been added to purified antithrombin in a 2:1 and 1:1 molar ratio respectively. The inner dense band was confined to slower migrating antithrombin-thrombin complexes that increased in precipitin area following the addition of more

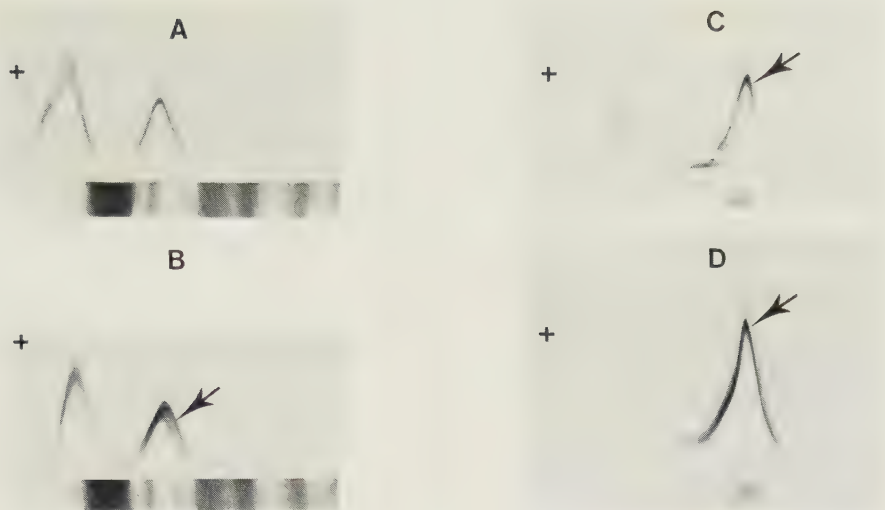


Fig. 5. Crossed immunoelectrophoresis
 A and B. Normal serum electrophoresed in presence of heparin and analysed with antisera No 1 and No 2 respectively. Arrow denotes precipitin loop produced by antithrombin-protease complexes.
 C and D. Purified antithrombin-thrombin complexes run in 2:1 and 1:1 molar ratios respectively in presence of heparin and analysed with antiserum No 2. Arrows denote distinctive precipitin loop of antithrombin-thrombin complexes.

thrombin.

Antiserum No 2 therefore clearly differentiates free antithrombin from antithrombin-protease complexes.

Immunochemical analysis of active and inactive antithrombin (I)

Immunological methods generally measure total antigen concentration and as such may not necessarily reflect functional activity. Individuals with reduced plasma antithrombin levels are predisposed towards thrombotic episodes (38,77). Therefore measurements reflecting the functional capacity of antithrombin are clinically most relevant. Immunochemical measurement of antithrombin levels have been extensively employed (78,79,80, 81). However, the results obtained have demonstrated both strong and poor

correlation with antithrombin functional activity.

These anomalies, plus our own discrepant results when two different antisera were tested, (see previous section), stimulated a study to elucidate requirements necessary to obtain clinically meaningful antithrombin values by electroimmunoassay.

The test samples used in this part consisted of plasma and serum taken from 30 blood donors plus plasma from 16 known antithrombin deficiency patients. Strong positive correlation was obtained between antithrombin activity, (heparin cofactor activity as measured by a chromogenic substrate assay - S 2238), and electroimmunoassay using both antisera ($r = 0.96$ and 0.98 respectively) when the results from the plasma samples only were compared. The addition of the results obtained with the serum samples, containing appreciable amounts of inactive antithrombin-protease complexes, significantly decreased the correlation obtained with antiserum No 1 but not with antiserum No 2. This discrepancy highlights the limitations of antisera which do not distinguish different molecular forms of antithrombin.

Serum contains both active and inactive forms of antithrombin but its total antigenic content approaches that of plasma. The second antiserum which distinguishes the two populations of antithrombin, provided results that correlated well with the functional capacity of antithrombin in the test samples. This interpretation was confirmed by the addition of increasing amounts of purified thrombin to two plasma samples with known antithrombin content.

The obvious quantitative disparity demonstrated between antisera No 1 and No 2 when plasma and serum samples were used indicates that a) the antiserum used should preferably be capable of producing results that reflect functional capacity of antithrombin and b) test samples for assay should preferably contain a minimum of inactive protease complexes. Consequently

fresh plasma (or plasma stored at -70°C for up to 6 months) is recommended.

Different binding sites on heparin exist for antithrombin and antithrombin-protease complexes (II)

Heparin binds to antithrombin and induces an accelerating rate at which the protein inactivates thrombin and other serine proteases of the coagulation cascade (33,82). Commercial heparin preparations are heterogeneous in this respect and can be further separated into two distinct fractions by affinity chromatography over matrix linked antithrombin. The fraction which has strongest affinity for antithrombin has greatest anticoagulant activity, the other fraction binds weakly to antithrombin and has low anticoagulant activity (83). Decreased binding of heparin to antithrombin occurs following the complex formation between antithrombin and thrombin (37).

This part of the study compared the binding of antithrombin and antithrombin-protease complexes to unfractionated heparin that was first immobilized on CNBr activated Sepharose. In the initial experiments whole human serum was applied to the heparin-Sepharose columns. After extensive washing the heparin-bound proteins were eluted with an increasing NaCl gradient. The elution patterns of antithrombin and antithrombin-protease complexes were monitored by the antithrombin antiserum capable of distinguishing between the two molecular species. Antithrombin-protease complexes were eluted between 0.2 and 0.65 M NaCl whereas the bulk of antithrombin (90 %) eluted between 0.7 and 1.2 M NaCl.

The next step was the separate application of excess purified antithrombin and excess purified antithrombin-protease complexes and the determination of their respective elution profiles. The patterns of elution were the same as observed with whole serum. The third step was to reapply the purified antithrombin preparations in excess amounts sequentially to the

same heparin-Sepharose column. Antithrombin-protease complexes were not displaced from heparin-Sepharose by subsequent application of excess loads of purified antithrombin as evidenced by the appearance of antithrombin only in the void volume. Eluted antithrombin proteins in all experiments yielded the same profile as for serum, i.e. antithrombin-protease complexes were removed from the heparin columns between 0.2 and 0.65 M NaCl; antithrombin between 0.65 and 1.2 M NaCl. It was therefore apparent that the two molecular species of antithrombin bound to different sites on the heparin molecule.

Isolation of active antithrombin (III)

The discovery that heparin interacts strongly with antithrombin (33) led to the use of heparin-affinity chromatography as an efficient first step in the purification of active antithrombin (84). Further chromatographic steps, however, are necessary to provide material of acceptable purity, even though the antithrombin binding sites on heparin appear highly selective (19). Heparin binding proteins other than antithrombin may contaminate the final product and possibly physically mask the specific antithrombin binding sites on heparin, thus limiting the maximum load of antithrombin which can be applied to the columns. Furthermore, serine proteases, such as Factors IX and X, also bind directly to heparin (85) and potentiate the formation of inactive antithrombin-protease complexes which contribute further to the heterogeneity of purified antithrombin.

The preceding section has demonstrated the existence of at least two types of protein binding sites on heparin-Sepharose. One is highly selective for antithrombin and the other is selective for antithrombin-protease complexes and certain other proteins. These findings, the identification of most proteins capable of binding to the major heparin fraction(s) plus earlier reports demonstrating that lipoproteins could be precipitated from

serum with heparin or dextran sulphate in the presence of divalent cations led to a simple initial precipitation step whereby virtually all heparin binding proteins other than antithrombin were removed prior to subjecting the test material to heparin chromatography.

Purification of antithrombin from various sources of test material, (plasma, serum and ascitic fluid), demonstrated that unless dextran sulphate treatment of the samples is used, antithrombin-protease complexes are formed during chromatography with reduced yields of antithrombin. The other heparin binding proteins not only contaminated the final antithrombin product, but they also decreased the column's capacity to bind antithrombin. These test samples disclosed the existence of at least three molecular forms of antithrombin with regard to heparin affinity: strong heparin affinity, reduced heparin affinity and a form which apparently has no affinity for heparin-Sepharose.

Dextran sulphate appeared to share with the low affinity high capacity fraction(s) of heparin the property of binding antithrombin-protease complexes and certain other plasma proteins. The finding that hyaluronic acid did not induce the same precipitating effects implies that sulphate groups play a role in this binding.

The use of dextran sulphate precipitation would therefore seem to be an important first step in the purification of active antithrombin to ensure consistently good yields (88-92 %) which are not contaminated with potentially hydrolysing proteases.

Conclusion

Antithrombin-protease complex formation is associated with structural changes on antithrombin which render such complexes antigenically distinct from the native molecule. Antithrombin levels measured immunochemically can

accurately reflect functional activity provided the antiserum used distinguishes between the free and complexed forms of the inhibitor protein. The fact that antithrombin and antithrombin-protease complexes have different binding sites on heparin would have been difficult to realize without the use of such antiserum. A recent report by Wallgren *et al.* (122) demonstrating that new antigenic determinants are created in modified antithrombin by a single peptide bond cleavage may explain these observations.

HEPARIN-C1 INTERACTIONS

Initial studies using affinity chromatography on heparin linked to CNBr-Sepharose (I) indicated that many complement factors from both classical and alternative activation pathways, including C1, interacted with heparin. Most of the earlier studies have utilized soluble polyanions (54,57). It was considered, therefore, that heparin-C1 interactions might better be explored using matrix-bound heparin and the reaction products obtained analysed directly by immunochemical methods. Electroimmunochemical analyses of C1 as utilized here, have been previously defined (86,87). It was on this basis that content, type and activity status of individual and complexed C1 or C1̄ inactivator components were made.

Binding of C1 subcomponents to heparin-Sepharose (II, V)

C1 binds to heparin-Sepharose via C1q. These findings confirm previous reports (54,55), and demonstrate that C1q-heparin binding was neither calcium nor temperature dependent. The zymogens C1r and C1s had no direct affinity for heparin but could be induced to bind to heparin in the presence of C1q. This was in direct contrast with their activated counterparts, C1̄r and C1̄s, as they bound directly to heparin. These findings appear analogous to those reported for prothrombin (88). Prothrombin does not bind to heparin at physiological salt concentrations, whereas thrombin binds heparin rather firmly. Earlier reports (55) have shown that heparin and other sulphated polyanions effectively inhibit the consumption of C4 and C2 by C1̄s. Rent *et al.* (56) and Takada and Takada (57) reported enhanced C1̄s inhibition by C1̄ inactivator in the presence of sulphated polyanions. The direct binding of C1̄s to heparin may induce not only a conformational change in C1̄s by heparin linkage, thereby increasing the affinity of C1̄s for C1̄ inactivator, but heparin binding might also physically mask the C4 binding site on C1̄s to

ensure the next step in the activation sequence does not occur, i.e. the formation of C3 convertase.

Activation, dissociation and assembly of C1 on heparin-Sepharose (V)

Activation of C1 is normally initiated through the binding of C1q to the Fc portion of IgG or IgM (46), whereby C1r and C1s are converted to active enzymes. The activity of C1 under physiological conditions is regulated by C1 inactivator which irreversibly binds C1r and C1s subunits, dissociating them from C1q and blocking further enzymatic activity (48,89). These investigations demonstrated that C1 can be activated, dissociated and reassembled on heparin-Sepharose (Fig. 5).

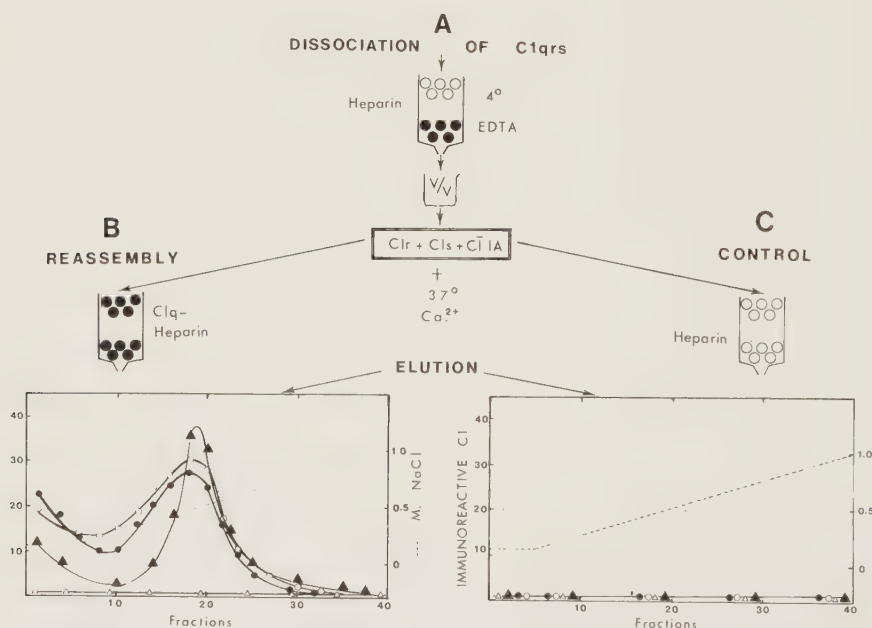


Fig. 5. Dissociation and reassembly of C1
 A) Dissociation of C1 in presence of 0.02 M EDTA.
 B) Reassembly of C1 on addition of C1r and C1s.
 C) Control experiment.

Activation of C1 on heparin-Sepharose occurred at 37°C in the presence of calcium. Approximately 60 % of applied C1r-C1s was retained on the heparin column via C1q. That activation occurred during heparin affinity chromatography was verified by the ability of C1r and C1s eluted from heparin-Sepharose to form complexes with purified C1 inactivator. This contrasted with experiments performed at 4°C where small amounts of C1 (20 %) were retained on heparin-Sepharose in the presence of calcium, however C1r-C1s remained in zymogen form. Considerable amounts of C1r-C1s (60 %) remained complexed to heparin-bound C1q in the presence of C1 inactivator which indicates that a lag phase for activation of C1r occurs and thereby allows C1 inactivator to pass C1 prior to its activation. A time lag of some 2-10 minutes has recently been shown where antigen-antibody complexes were used to study C1 activation mechanisms (90). Activation of C1 in guinea pig serum using sulphated dextrans occurred within 30 minutes (59). However, neither the latter report nor these heparin affinity studies have determined the efficiency or mechanisms by which C1 can be activated by sulphated polysaccharides. What can be said is C1 activation was induced via C1q, as C1r-C1s activation did not occur during their passage over heparin-Sepharose in the absence of C1q (Fig. 5 C). Heparin-Sepharose could, therefore, be considered as a possible medium by which to study further the mechanisms of antibody independent complement activation.

Macromolecular C1 is dependent on calcium for its integrity (40). Consequently, the utilization of chelating agents can effectively dissociate C1, leaving C1q retained on heparin-Sepharose with C1r-C1s being recovered in the void volume. Significant, albeit partial, dissociation occurred in the presence of calcium in a temperature dependent manner. At 4°C, 80 % of C1r and C1s were dissociated from C1q and recovered as zymogen complexes in the void volume. At 37°C however, 40 % of C1r and C1s were dissociated from C1q

and were collected as C1 inactivator complexes in the void fractions. Increased retention of C1r-C1s on heparin bound C1q at 37°C compared to 4°C probably reflects a greater affinity of enzymatically active C1r for C1q. Nevertheless, the mechanisms by which heparin apparently dissociates C1 remain to be determined.

Assembly of C1 was effected on heparin-Sepharose at 37°C in the presence of calcium, inducing activation of C1r-C1s during the process (see Fig. 5).

Influence of C1t on C1s-heparin binding (VI)

C1t is a normal plasma protein that shares with CRP structural similarities and calcium dependent ligand binding characteristics (91). C1t was described as a novel protein, detected in EDTA eluates obtained from columns of IgG linked to Sepharose (92), and in the euglobulin fraction of serum (93). Initial studies implicated C1t as a fourth subcomponent of C1 serving as a bridge between C1s and C1q via a calcium dependent bond (92). Further investigations failed to confirm these findings as its selective removal had little effect on the haemolytic properties of C1 in serum (93,94). The strong calcium dependent binding of C1t to sulphated agarose or heparin has been exploited for its purification (95). Although no longer thought to be related to C1, isolated C1t has recently shown to be capable of binding to fixed complement *in vitro* (91).

During studies of heparin-C1s interaction, one preparation demonstrated strong affinity for heparin-Sepharose in the presence of calcium (Fig. 6 A). Further investigation revealed this preparation to be contaminated with C1t. Purified C1s subjected to chromatography in the absence of C1t consistently failed to bind to heparin-Sepharose (Fig. 6 B). The heparin affinity of C1s in C1t containing preparation was apparently due to a C1s-C1t complex which was firmly bound to heparin via C1t. The significance of such a complex has

yet to be resolved. One possible explanation is that heparin potentiates the binding of Clt to Cls. That 1.5 M NaCl in the absence of chelating agents failed to elute Clt from heparin-Sepharose indicates that Clt binds more firmly to heparin than does antithrombin (84).

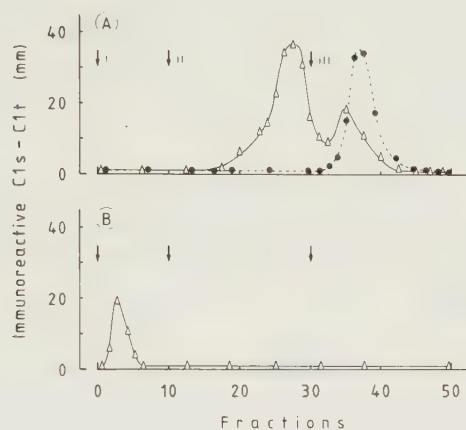


Fig. 6. The influence of Clt on Cls-heparin binding
 A) Elution of Cls (Δ-Δ) from heparin-Sepharose in presence of Clt (●-●).
 B) Cls (Δ-Δ) applied to heparin-Sepharose in the absence of Clt.

Isolation of C1r and C1q by heparin affinity chromatography (VI, VII)

Recent evidence indicates that multiplicity of binding of C1q to the C_H2 domain of IgG is essential for C1 activation and that local antibody concentration is critical for lysis (96). Activation of C1 occurs only when all three subcomponents and calcium are present (40). C1r provides not merely a link between C1q and C1s but appears to be the rate limiting step by which C1 is activated. A conformational change in C1r has been proposed whereby a catalytic site in the molecule is exposed which in turn induces activation of other C1r molecules (40,47).

Zymogen C1r was purified by two sequential, heparin affinity chromatography steps at 0°C. Macromolecular C1 retained on heparin-Sepharose during the first step was dissociated from C1q by a Tris-HCl-citrate buffer. The pooled, recalcified C1r containing fraction was applied to, and retained on a fresh heparin-Sepharose column equilibrated at a lower conductivity (15 mS/cm). Extensive washing of this column followed by dissociation of C1s and C1t, using the citrate buffer allowed pure, zymogen C1r to be recovered from the column with an NaCl gradient. The zymogen form of the eluted C1r was verified by its inability to form complexes with purified C1̄ inactivator and by its ability to form C1r-C1s complexes without inducing the activation of C1s.

Purification of C1q from five different animal species was achieved by a relatively simple, two-step chromatographic procedure. The first step utilized heparin-Sepharose in the presence of EDTA and appeared to have some advantages over the more widely used euglobulin fractionation procedure of previous reports (97,98). Proteins that co-precipitate with C1q during euglobulin fractionation did not bind to heparin-Sepharose in the presence of EDTA (i.e. C1t, CRP, C1r or C1s). Furthermore, IgG, a major contaminant of C1q preparations (99), did not bind to heparin-Sepharose at 0.15 M NaCl.

The second and final step used the Clq-IgG interaction. The Clq-rich fraction eluted from heparin-Sepharose was applied to rabbit-IgG conjugated Sepharose. Application of an increasing NaCl concentration gradient eluted Clq, the only detectable protein that bound to rabbit-IgG. Recoveries of purified Clq were between 84 and 95 %. The observation that Clq precipitated directly in agarose gels during conventional zone electrophoresis proved to be highly selective for Clq but not species-specific. This reaction was therefore used to advantage to rapidly monitor and quantitate Clq from the five species tested. Purified Clq isolated from all species retained its functional capacity as determined by their ability to form haemolytically active C1 when added to Clq depleted normal human serum.

Conclusion

Heparin conjugated Sepharose has been utilized as a medium to demonstrate antibody independent complement activation. This affinity system can also be used to study dissociation and reassembly of C1, and has been exploited as a crucial step in the purification of Clq and zymogen Clr.

AGAROSE-PLASMA PROTEIN INTERACTIONS

Agarose has the physicochemical properties approaching those required to produce the ideal gel matrix for separation of biopolymers corresponding to charge differences. The gel acts as an anticonvection medium that can be made biologically inert or to contain controlled ionic properties (100). Hjertén (101) and Låås (102) have demonstrated that charged groups within the agarose gel matrix induce electroendosmosis and may cause non-specific adsorption of high molecular weight polyelectrolytes. Little consideration appears to have been given as to the effect these charged groups in agarose preparations may have on direct protein binding and the consequent influence on formation, migration, and solubility of antigen-antibody complexes.

The first section below deals with various types of direct interactions which occur between agarose and proteins and are exemplified with four different, purified plasma protein systems. The second part considers the role that sulphate groups affixed to the agarose gel matrix have on immunoprecipitation of one of these proteins - antithrombin. The third and final part of this section demonstrates the usefulness in exploiting the highly selective agarose gel-Clq precipitation reaction which occurs during conventional electrophoresis.

The interaction of certain proteins with the agarose gel

The interaction of plasma lipoproteins with sulphated polyanions and agar gels is well documented (26,103). Low density lipoproteins can be precipitated out of solution by heparin or sulphated dextrans in the absence of divalent cations. High density lipoproteins, by contrast, require high concentration of both sulphated polyanions and divalent cations to precipitate (29).

This section illustrates a spectrum of similar phenomena that occur in agarose gels during electrophoresis. Four purified plasma proteins other than lipoproteins were selected on the basis that they bound to heparin rather firmly, i.e. antithrombin, thrombin, Clt and Clq. The same agarose was utilized throughout these experiments. It contained little sulphate (SO_4^-) (0.22 % w/w) with no detectable pyruvate. The migration distance of the different proteins in individual electrophoretic runs were related to albumin, in order that interactions and migration distance of individual proteins could be compared.

Antithrombin (Fig. 7 A) demonstrated weak but consistent trailing in a calcium independent manner due to interaction with the agarose. The inclusion of phosphate into the buffer (20 mmole/L) was sufficient to prevent this interaction. Heparin (0.05 mg/ml of gel) when added to the gel linked firmly with antithrombin and in doing so inhibited the interaction between antithrombin and the gel inducing a profound increase in antithrombin's migration distance.

Thrombin (Fig. 7 B) demonstrated strong gel interaction that depended upon calcium, requiring 20 mmole/L EDTA in the gel buffer before the interaction was prevented completely. Heparin (0.05 mg/ml), when added to the calcium-containing gel induced a marked increase in the electromigration of thrombin.

Clt demonstrated very strong, calcium dependent interaction with the gel and required 90 minutes at 20 V/cm before entering the gel completely (Fig. 7 C). In the presence of 2 mmole/l EDTA, this protein migrated as a distinct α_1 band. Heparin (0.05 mg/ml) when incorporated into the gel failed to inhibit the gel interaction.

Complement subcomponent Clq (Fig. 7 D) demonstrated an intense opaque

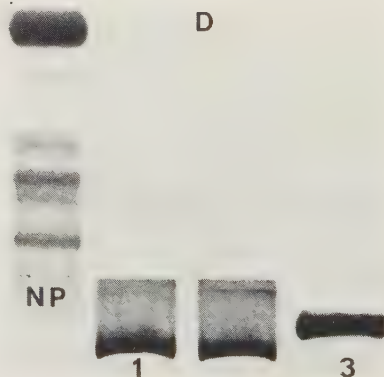
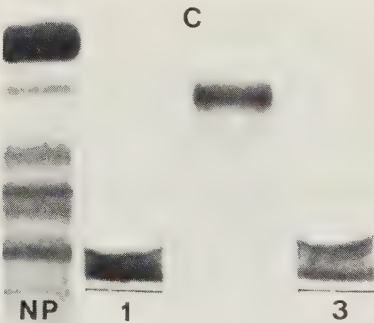
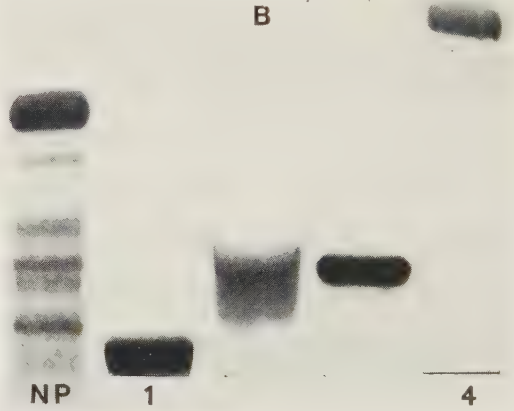
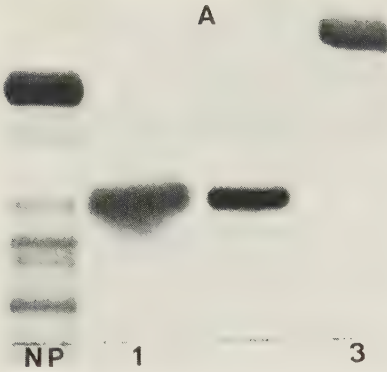
Fig. 7

- A) Antithrombin (AT). Electrophoresis 20 V/cm. NP normal plasma. 1:15 for reference.
- 1) AT run in 0.075 M barbital buffer + EDTA pH 8.6.
 - 2) AT run in 0.3 M barbital buffer + 0.02 M (O + pl) 7.0.
 - 3) AT run in same conditions as 1) + heparin (25 IU/ml).

- B) Thrombin electrophoresis 20 V/cm.
- 1) Thrombin run in 0.075 M barbital + 0.02 M Ca^{2+} .
 - 2) Thrombin run in 0.075 M barbital + 0.02 M EDTA.
 - 3) Thrombin run in 0.075 M barbital + 0.2 M EDTA.
 - 4) Thrombin under same conditions as 1) + heparin (25 IU/ml).

- C) Clt electrophoresis 20 V/cm.
- 1) Clt run in 0.075 M barbital buffer and Ca^{2+} pH 8.6.
 - 2) Clt run in 0.075 M barbital buffer and EDTA.
 - 3) Clt same conditions as in 1) but in the presence of heparin (25 IU/ml).

- D) Clq electrophoresis 20 V/cm.
- 1) Clq run in 0.075 M barbital buffer + EDTA.
 - 2) Clq run in 0.075 M barbital buffer + heparin.
 - 3) Clq run in 0.03 barbital buffer + 0.2 M PO_4 (pH 7.0).



precipitate that commenced from the application slit as a cathodal migrating zone, which could be observed directly in the gel before fixation. This gel interaction was calcium independent and could not be inhibited by the inclusion of heparin into the gel (0.05 mg/ml). Phosphate containing buffers provided they were at a pH of 7.0 to 9.0 converted cathodal precipitation to a discrete homogeneous slowly migrating cathodal protein band.

Matrix-bound sulphate groups are required for enhanced immunoprecipitation of antithrombin (I, IV)

During the course of elucidating practical requirements for assaying immunoreactive antithrombin levels, spurious results were obtained that were apparently related to different batches of agaroses. The anomalies stimulated further studies to determine a) the influence charged groups fixed in the gel have on the development and precipitation of antithrombin-antibody complexes, and b) whether polyanions, when added to the test systems, could effect antithrombin immunoprecipitation.

Utilization of four batches of agaroses, differing principally in their sulphate content, revealed that the more sulphated agarose gels gave immunochemical values for antithrombin which reflected better functional activity. Desulphated gels produced immunochemical results which correlated poorly to functional activity.

The same test samples produced smaller radial immunodiffusion precipitin ring sizes but larger EIA precipitin loops in desulphated gels than in gels with higher sulphate content. These differences suggest that the sulphate groups influence the precipitation of the antigen-antibody complexes. Subjecting gels to electrophoresis for 24 hours prior to testing did not influence the results indicating that charged groups affixed to the gel were

responsible for the above effects.

Recovery studies were undertaken whereby ^{125}I -labelled antithrombin was added to test samples before subjecting them to single radial immunodiffusion and electroimmunoassay analysis. Immediately following the required reaction times the gels were pressed and washed extensively. The dried gel films were subjected to autoradiography, after which individual immunoprecipitates were cut from the gel and the amount of radioactivity measured. Comparative analysis of the retained radiolabelled antithrombin in the gels revealed a marked reduction in the recovery of antithrombin from gels with low sulphate content (Fig. 8 A).

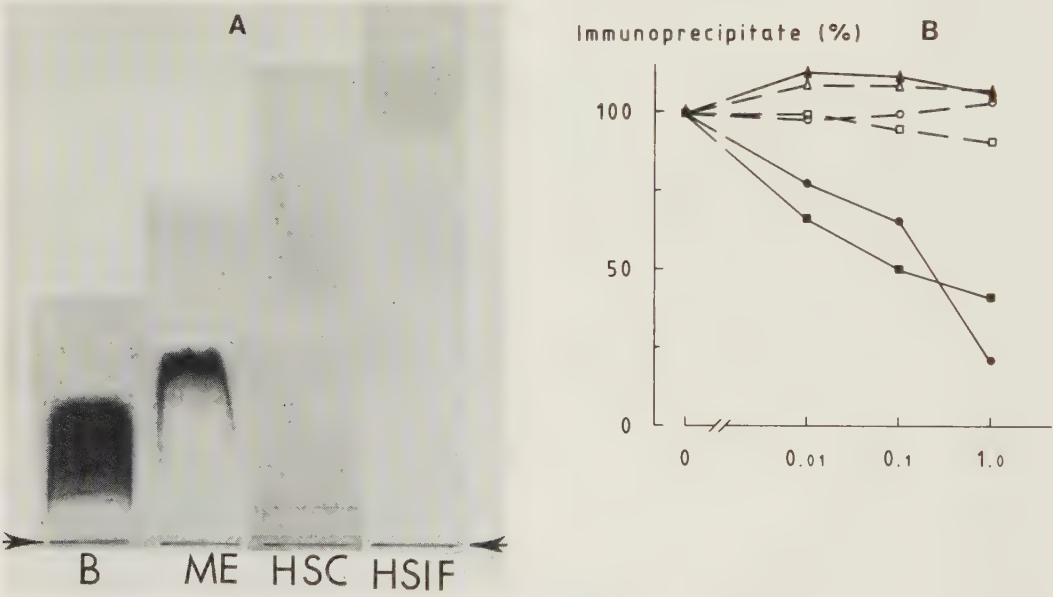


Fig. 8. A) Autoradiography of antithrombin immunoprecipitates following electroimmunoassay in gels with decreasing SO_4^- content, illustrating the poor recovery of immunoprecipitates in the desulphated gels HSC and HSIF.
B) The effect of adding increased concentrations (0.01-1.0 mg/ml) of heparin (●-○), dextran sulphate (■-□) or hyaluronic acid (▲-△) and the % recovery of immunoprecipitates of antithrombin (solid symbols) and α -1-proteinase inhibitor (open symbols).

The addition of heparin and/or dextran sulphate to test systems induced a moderate to marked reduction of antithrombin recovery which was dependent on the concentration of polyanion (Fig. 8 B). Hyaluronic acid when added to the gel in the same concentrations, however, did not inhibit antithrombin immunoprecipitation. A related plasma protein, α -1-antitrypsin, which does not interact with agarose or have affinity for heparin was also subjected to the same experimental rigors. Recovery analysis here demonstrated that this protein was influenced by neither the quality of the gel nor by the addition of polyanions.

Precipitation of Clq in agarose gels (II, V, VII)

Agarose gel-Clq interactions were observed during the initial investigations undertaken to identify the various plasma proteins which linked firmly to heparin-Sepharose. This interaction was recognised as a characteristic, tongue-like precipitate that stretched cathodally from the site of application (see Figs 3, 4 and 5 in article I). Its appearance was immediately apparent, following electrophoresis and did not require fixation to be retained in the gel.

Precipitation of Clq in the gels was not calcium dependent, but could be prevented if the ionic content of the gel buffer was increased to 250 mM/L. Agarose with higher sulphate content induced greater intensity of Clq precipitation while simultaneously retarding its cathodal migration. This implied that precipitation was related to the content of sulphate groups in the gel. Prerunning the agarose gels by electrophoresis for 24 hours prior to testing did not alter appreciably the observed Clq-gel precipitation results, indicating that charged groups fixed to the gel matrix were more likely responsible.

Comparative double diffusion analyses, using Ouchterlony type patterns

in 1 % (w/v) agarose gels, induced the formation of precipitin-like bands between heparin and Clq, dextran sulphate and Clq - but not between hyaluronic acid and Clq. The reaction between dextran sulphate and Clq could be directly visualized within 8 hours of diffusion, whereas the heparin-Clq interaction required at least 24 hours. These bands disappeared when the gels were washed in NaCl solutions with a concentration of more than 50 mM.

Single radial diffusion of purified Clq in agarose gels led to the formation of opaque, precipitin-like rings around the application wells with diameters proportional to the concentration of Clq. The length of the precipitin-like track produced with Clq in agarose gels during electrophoresis was also shown to be linear with Clq concentrations between 50 and 400 $\mu\text{g/ml}$ provided electrophoresis at 20 V/cm continued for 1 hour (Fig. 9).

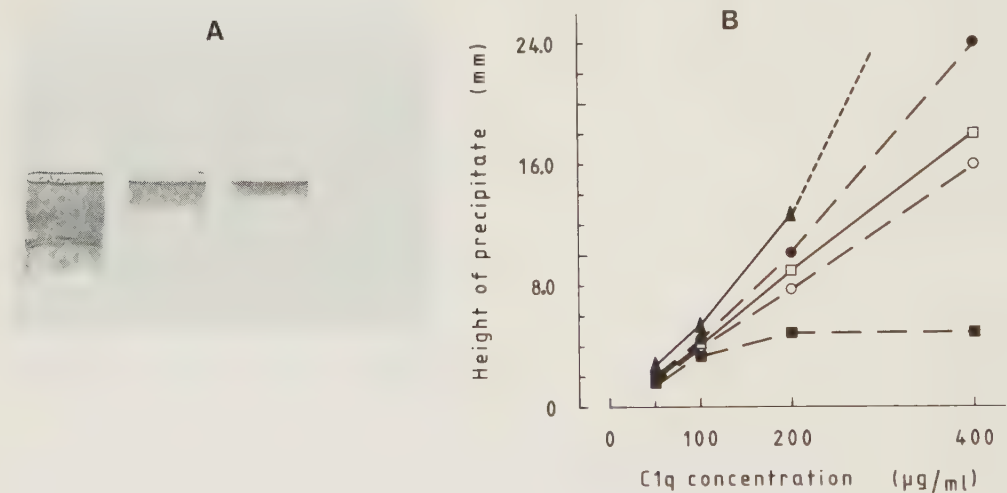


Fig. 9. Clq precipitation in agarose gels.
 A) 10 μl samples of purified Clq in concentrations of 400, 200, 100 and 50 $\mu\text{g/ml}$ electrophoresed for 1 hour at 20 V/cm.
 B) Effect of running time on Clq precipitation in gels.
 ■-■ 15 minutes; ○-○ 30 minutes, □-□ 1 hour; ●-● 1½ hours; ▲-▲ 2 hours.

The developed complexes, however, were not stable during prolonged electrophoresis as dissociation was observed if electrophoresis was continued more than 90 minutes.

The electrophoresis approach was used to rapidly monitor Clq-rich fractions during Clq purification from five different animal species. This technique also provided quantitative data concerning the amount of Clq recovered from each isolation step and precluded the need to have available species-specific anti-Clq antiserum. These results also imply that Clq-gel interactions reflect a functionally active component as heat-treated and/or non-IgG binding Clq failed to produce the same precipitin-like interaction.

Conclusion

Contaminating sulphate groups in agarose contribute significantly to the binding of certain (heparin interacting) plasma proteins. Such interactions can have a profound influence on immunoprecipitation, e.g. antithrombin, and therefore need to be considered when rationalising practical requirements to optimize electroimmunochemical assays.

BIOLOGICAL IMPLICATIONS

Although heparin is best known for its anticoagulant activity, it also demonstrates antiinflammatory activity and is capable of inhibiting delayed hypersensitivity reactions (104). Heparin is stored and synthesized within mast cells which are found in all organs rich in connective tissue (105). In virtually all instances mast cells are located in close proximity to the capillary blood vessels (106). Release of heparin from the mast cell may occur in certain pathological situations, eg. systemic mastocytosis (107) but heparin probably does not occur in the circulation under physiological conditions (108).

Evidence that a very small fraction of heparin is involved in anti-thrombin binding has been provided by Thaler and Schmer (109) who demonstrated that 3 % of the heparin conjugated to agarose was active in binding antithrombin. Lindahl and colleagues (19,20) have recently isolated a strongly antithrombin-binding fragment from heparin which corresponded to 2-3 % of a high activity heparin fraction. Bengtsson *et al.* (110), and Niewiarowski *et al.* (111) have demonstrated that the heparin binding sites selective for lipoprotein lipase and platelet anti-heparin proteins are different from those that interact with antithrombin.

This investigation (I) has demonstrated that heparin conjugated to Sepharose primarily binds a select group of plasma proteins other than antithrombin. Less than 10 % of the immobilized heparin interacts directly with antithrombin. The majority of plasma proteins which bind to this larger, low affinity high capacity fraction of heparin can be divided into four functionally related categories; coagulation proteins, complement proteins, protease inhibitors and cell surface proteins. Virtually all the heparin-interacting proteins can be removed from plasma by precipitation with dextran sulphate (III). This implies that not only heparin and dextran sulphate but also

other sulphated polysaccharides, such as heparan sulphates or dermatan sulphates, may display similar binding effects. That non-sulphated hyaluronic acid was unable to effect similar precipitation indicates that the sulphate groups are most likely responsible.

These results suggest that heparin or, more importantly, heparanoids, may be directly involved in modulating important biological processes within the extracellular fluids. In addition, most sulphated glycosaminoglycans, except heparin, have been identified within the arterial intima (112). Consequently, these polysaccharides may not only be accessible to circulating plasma components but they may also provide selective binding of these components to vessel walls. The recovery of surface-linked proteins from heparin conjugated to Sepharose (I) indicates that biological competition may occur between these proteins for the low affinity, high capacity binding sites of heparin(oids) in tissues. Cellular glycosaminoglycans have been implicated in binding fibronectin to cell surfaces (113). Sulphated glycosaminoglycans, in addition, enhance the affinity of fibronectin for collagen (114,115).

The remarkable enrichment of specified complement proteins obtained by heparin-affinity chromatography (I) may be indicative of an ordered process in which heparin(oids) may interact directly with them and thereby exert some control over related processes. Other workers have shown complement-sulphated polyanion interactions that depended upon its molecular weight and degree of substitution with sulphate groups (54,59,116). Figure 10 illustrates the complement activation sequence with proposed steps where heparin may exert its effect. The present studies involving heparin-C1 interaction (I, V, VI) in conjunction with other reports indicate that the C1 macromolecule can be directly inactivated by heparin(oids) dissociating C1q from C1r and C1s. However, activation of C1 was also demonstrated at 37°C. Enzymic C1r and C1s bind directly with heparin(oids) and this potentiates

their inactivation by $C\bar{I}$ inactivator. Whether heparin has catalytic action whereby one heparin molecule can promote the activation of several $C\bar{I}$ r or $C\bar{I}$ s molecules, as proposed for heparin-antithrombin-thrombin interactions (33,36), remains to be elucidated. Strunk and Colten (60) have recently shown that heparin prevents the cleavage of C4 and C2 by $C\bar{I}$, and C2 is one hundredfold more sensitive to heparin inhibition than C4. Our results suggest this might be due to direct C2-heparin binding that physically

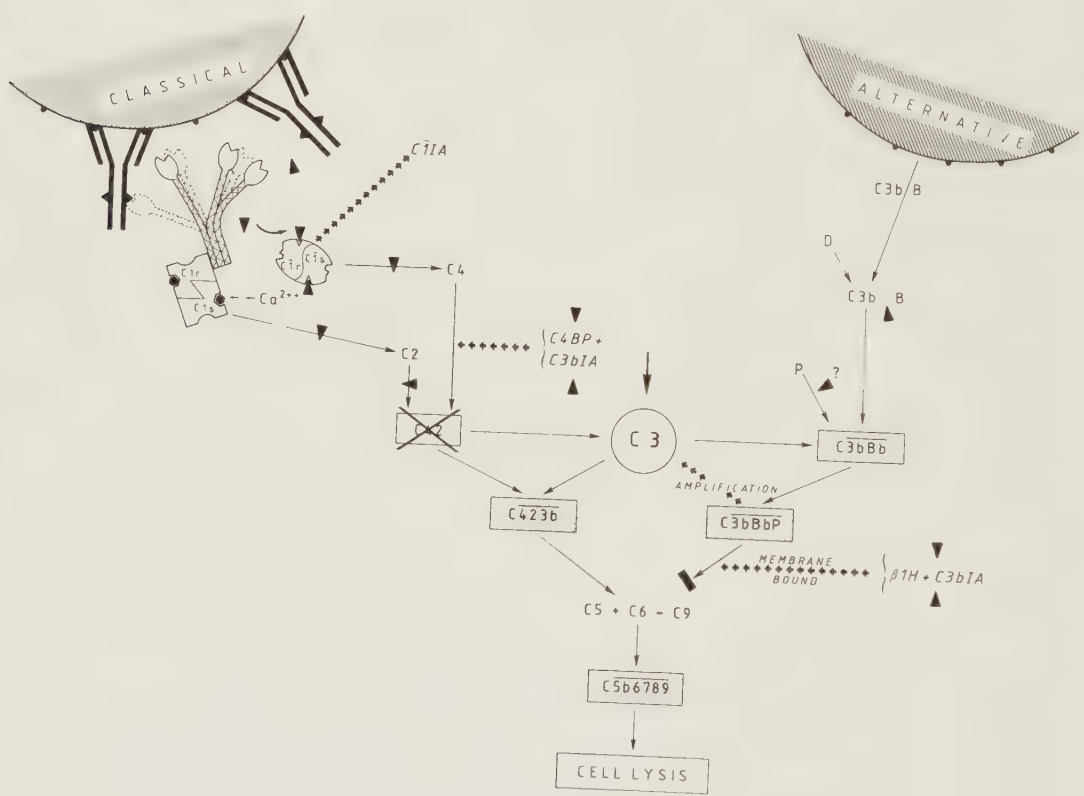


Fig. 10. Proposed steps whereby heparin can exert its effect on the complement cascade. Heparin is denoted by (▼); broken heavy arrows indicate enhancement of interactions.

prevents C1s-C2 interaction from taking place. Chondroitin-4-sulphate, previously recognised, naturally occurring inhibitor of C1q (117,118) has recently been described (119). This represents the only demonstrable sulphated glycosaminoglycans found normally in blood (9).

Control of the amplification phase of the alternative pathway occurs with cleavage of C3b via C3b inactivator (51) which is facilitated by the binding of β 1H to C3b (120). The Bb fragment in C3bBb is displaced by β 1H even when the complex is stabilized by properdin (120). Sulphated polyanions have previously been shown to activate the alternative pathway (59). Direct binding of factor B, C3b inactivator and β 1H to heparin-Sepharose (1) suggests that heparin may not only activate these steps in the complement sequence but it may also be capable of effecting modulation at its central point, i.e. C3b or C3 convertase. Kazatchkine and colleagues (121) demonstrated that heparin, when attached to a particle surface, promoted the interaction of bound C3b with β 1H and C3b inactivator, suppressing further activation of cell bound C3. In contrast, Bitter-Suermann *et al.* (116) recently reported that particulate sulphated dextran was capable of binding reversibly to guinea-pig and human β 1H. This fixation led to an efficient fluid phase activation of complement. These results suggest there may be two independent mechanisms for control of the alternative pathway; one for surface bound components and another for fluid phase activation.

Sulphated polysaccharides bind to plasma proteins under a variety of conditions. Consequently, continued analysis concerning such behaviour, is likely to reveal further interaction of biological importance.

SUMMARY

Sulphated polysaccharides are linear polyanions, many of which have colloidal characteristics serving structural functions. In the search for other structure-functional relationships it has become increasingly apparent that the direct binding of plasma proteins to sulphated polyanions may have an important biological role. This investigation has studied some aspects of plasma protein sulphated polyanion interactions with particular reference to antithrombin and complement binding to heparin and/or agarose sulphate.

Antithrombin undergoes structural alterations when complexed with its natural proteases. The consequence of this interaction is the emergence of distinctive antigenic determinants on the inhibitor protein. An anti-serum was produced which could distinguish between active antithrombin and inactive antithrombin-protease complexes. Utilization of this antiserum demonstrated the existence of two protein binding sites on heparin - one highly selective for active antithrombin (high affinity, low capacity), the other more non-selective for antithrombin-protease complexes and a certain group of plasma proteins (low affinity but high capacity). Immunoreactive antithrombin levels could reflect functional activity, provided the above antiserum was used, even in the presence of appreciable amounts of inactive inhibitor protease complexes.

A single two-step procedure for the purification of antithrombin was developed. Most other heparin interacting proteins were first removed from the chromatography sample by dextran sulphate precipitation. This simple step allowed maximum loads of antithrombin to be recovered from a second step using heparin-affinity chromatography.

Studies concerning the binding of C1 to heparin conjugated Sepharose demonstrated that C1 could be activated, dissociated and reassembled on heparin columns. The C1 was retained on heparin-Sepharose in a calcium

dependent manner, via Clq. Zymogens Clr and Cls did not bind directly to heparin-Sepharose whereas their activated counterparts did. Cls in the presence of Clt, however, was firmly retained on heparin-Sepharose probably due to the formation of Cls-Clt complex. C1 inactivator and its complexes did not bind to heparin. Simple procedures for purifying Clq and zymogen Clr were devised illustrating the efficiency by which heparin-Sepharose can be used.

Sulphate groups affixed in agarose gels contribute significantly to the binding of certain (heparin interacting) plasma proteins. Such interactions can have a profound influence on immunoprecipitation as was exemplified using antithrombin. Clq precipitation in sulphated agarose gels was exploited to produce quantitative data during its purification from the plasma of five different animal species.

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Immunochemical Analysis of Active and Inactive Antithrombin III

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SUMMARY. Antithrombin III (AT) levels from normal and AT deficiency persons were measured by electroimmunoassay (EIA) and the results compared with a chromogenic assay (S2238). Discrepant results were obtained when plasma and serum were compared using one antiserum, and therefore did not always relate to functional activity. Another antiserum, however, when used was capable of differentiating active AT from inactive AT complexed with its proteases and demonstrated close correlation with all samples tested ($r=0.97$). The specificity of the antisera and consequent anomalous results were elucidated when purified human thrombin was added to plasma samples and subsequently reanalysed. Quantitative differences observed when serum samples were compared by single radial immunodiffusion and electroimmunoassay with one antiserum, illustrates the fundamental principle differences between the two methods. These results give some insight as to why previous anomalies with AT immunoassays have occurred. They also indicate that plasma and *not* serum should be used as clinical test material. AT antisera should be capable of recognizing and distinguishing free AT from AT/protease complexes if the results obtained by electroimmunoassay are to correlate with functional activity.

Individuals who have reduced plasma levels of antithrombin III (AT) have a tendency to thrombotic disease (Egeberg, 1965). Clotting assays initially utilized for measuring AT activity proved rather time consuming with poor reproducibility (Abildgaard *et al*, 1970). The development of specific immunochemical procedures by Fagerhol & Abildgaard (1970) permitted a relatively simple and reproducible approach to measure AT concentrations. Many workers have reported close correlation between immunoreactive AT and functional AT levels (Fagerhol & Abildgaard, 1970; Hedner & Nilsson, 1973; Damus *et al*, 1975; Marciniak & Grockerman, 1977). Collen *et al* (1977) considered immunoassays capable of detecting AT 'neoantigens' (which emerge after complexing with its natural proteases) to be useful in pinpointing *in vivo* activation of the coagulation cascade. However Schwick *et al* (1973) have reported there is little difference between immunoreactive AT levels in plasma and its corresponding serum. According to Andersson *et al* (1977) immunoassays of AT measure the sum of native AT plus AT contained as inactive AT/protease complexes and therefore

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immunoassay results do not correspond to biological assays. Bounameaux *et al* (1978) considered the absence of correlation between functional and immunochemical AT assays observed in people taking oral contraceptives was indicative that these methods measured different parameters.

These anomalies, plus our own discrepant results, when two different AT antisera were tested, prompted a study to rationalize what requirements were necessary to optimize immunochemical assays, particularly electroimmunoassay.

MATERIALS AND METHODS

Standard Plasma

Plasma samples collected in EDTA Na₂ from 10 healthy blood donors on the same day were pooled, aliquoted into small samples and stored at -70°C in plastic tubes. This plasma pool was given an arbitrary figure of 100% AT activity for both immunochemical and chromogenic substrate assays.

Test Samples

Blood samples from three healthy laboratory personnel were used for initial experiments. Blood was collected into preservative-free heparin, EDTA Na₂ and as serum. EDTA plasmas and corresponding sera were later collected from 30 blood donors and stored in small separate aliquots at -70°C until tested. Plasma samples from 16 known AT deficiency patients were also compared. Two plasma samples which had previously demonstrated 80% and 45% AT activity respectively were reanalysed following the addition of increasing amounts of purified human thrombin to individual aliquots of each sample.

Reagents

Antithrombin was isolated from normal citrated human plasma according to Miller-Andersson *et al* (1974). Antithrombin/protease complexes were obtained from normal human serum and purified as previously described (McKay & Laurell, 1980). Human prothrombin was obtained from a barium precipitate of normal citrated plasma and purified according to Stenflo (1976) while human thrombin was prepared according to Lundblad *et al* (1976).

Antithrombin III antisera. Rabbit antisera from two different sources were compared—one supplied by Nyegaard Laboratories, Oslo, Norway (Antiserum No. 1); the other was prepared in this laboratory by subcutaneous injections of 200 μg of purified AT in complete Freund's adjuvant at fortnightly intervals (Antiserum No. 2).

Chromogenic substrate (S2238) was kindly supplied in complete kit form (COATEST®) by Kabi, Stockholm, Sweden.

Agarose (ME) with medium electroendosmosis ($m^r=0.20$) was purchased from Marine Colloids Rockland, U.S.A. (Lot No. 50030).

Heparin was purchased from Vitrum Laboratories, Stockholm, Sweden, with a specific activity of 5000 IU/ml.

Buffer. Sodium barbitone/barbituric acid pH 8.6 (0.075 m/l) was used throughout.

Immunochemical Methods

Four methods used—double immunodiffusion according to Ouchterloney (1967), single

radial immunodiffusion (Mancini *et al*, 1965), electroimmunoassay (Laurell, 1972) and crossed immunoelectrophoresis (Ganrot, 1972).

Comparative analysis of AT levels. 76 samples of plasma and serum were assayed by electroimmunoassay using both antibodies separately and compared to the results obtained using the chromogenic substrate method according to Abildgaard *et al* (1977).

Calculations. Regression lines were calculated by the method of the least squares.

RESULTS

Ouchterlony. Both antisera produced single precipitating lines of identity against purified AT, AT/protease complexes, normal plasma and serum. Cross reactivity against purified thrombin or prothrombin in concentrations up to 0.5 mg/ml were not demonstrated.

Electroimmunoassay. Antiserum No. 1 (Fig 1a) demonstrated little variation in line appearance or peak heights using the same test samples, taken as EDTA, heparin or as serum. Antiserum No. 2 (Fig 1b), however, demonstrated considerable variation when the same series of samples was tested. Heparin samples produced peak heights 25% greater than the corresponding EDTA samples which in turn were 30% higher than the serum samples. Antiserum No. 2 also induced an additional dense inner precipitin peak with all serum samples which gave an obvious characteristic immunoprecipitation pattern.

Single radial immunodiffusion. Precipitin rings induced after 30 h incubation with antiserum No. 1 (Fig 1c) were of similar size when plasma samples taken in heparin or EDTA were tested. However, corresponding serum samples produced a more dense precipitate whose area was usually approximately 25% less than with plasma samples. Antiserum No. 2 (Fig 1d) produced

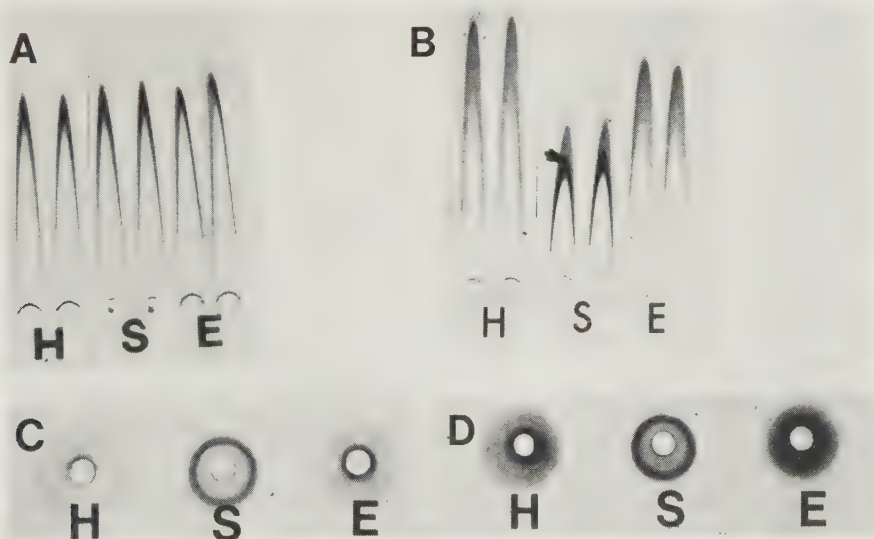


FIG 1. Immunochemical results obtained by electroimmunoassay and SRID using different antisera. H, S and E denote identical 10 μ l samples previously collected from one person into heparin, as serum, and in EDTA. A and C illustrate the results obtained with antibody No. 1. B and D illustrate the results obtained with antibody No. 2. Electroimmunoassay was run for 3 h, 12 V/cm. The arrow indicates the additional characteristic, inner, dense precipitin line produced in all serum samples.

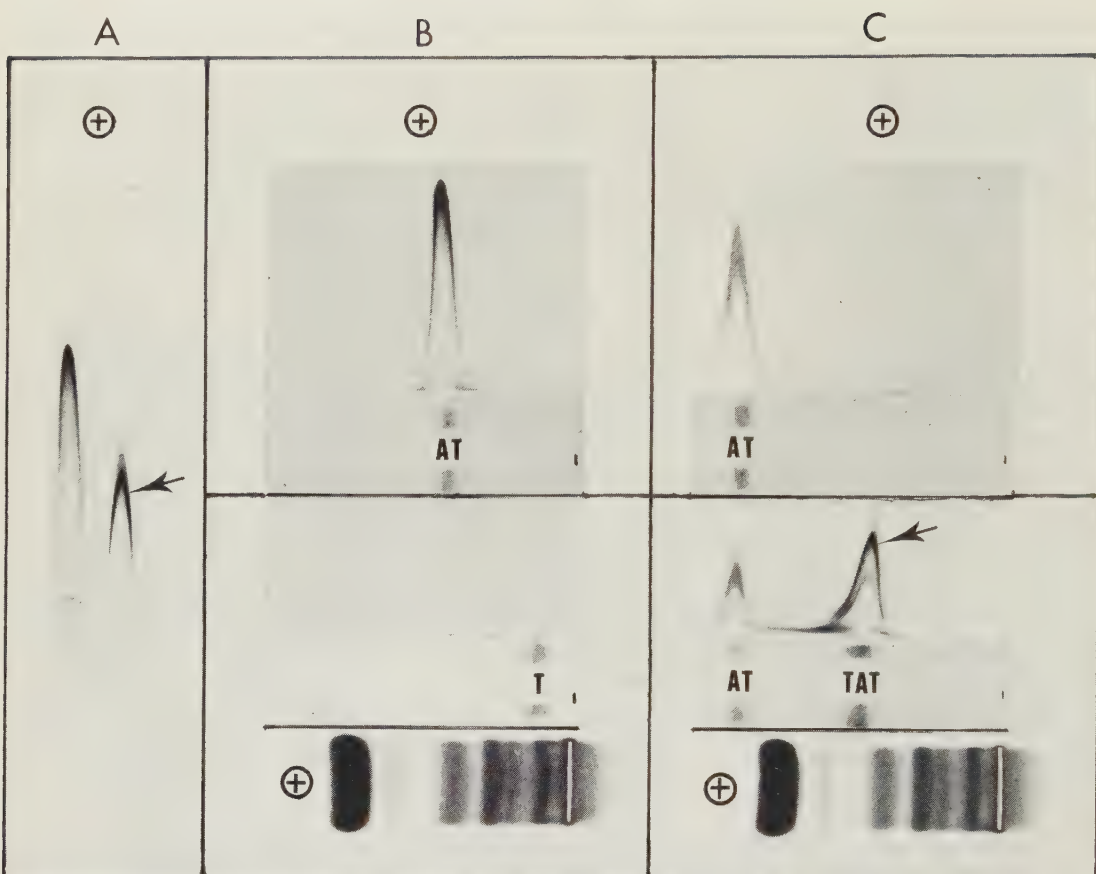


FIG 2. Immunochemical differentiation of antithrombin (AT) and AT/thrombin (TAT) complexes by antiserum No. 2. (A) demonstrates a single electroimmunoassay precipitin peak with purified AT (5 µg/ml). The addition of thrombin (500 IU/ml) to AT induced a reduction in height of the AT precipitin peak with the formation of an additional inner dense precipitin arc (denoted by arrow). (B) demonstrates a single α_2 precipitin arc with purified AT (5 µg/ml) by crossed immunoelectrophoresis. Thrombin (T) under these same conditions migrated as a β globulin but did not react with this antiserum. Normal plasma (1:10) is provided for reference (C). AT increased its electromigration to a single prealbumin peak when heparin (20 IU/ml) was incorporated into the gel of the first dimension in electrophoresis. This phenomenon allowed its separation from TAT complexes formed after the addition of thrombin (500 IU/ml) to AT (5 µg/ml). The newly formed dense precipitin arc was confined to the slower migrating TAT complex (denoted by arrow) and demonstrated partial immunological identity. Normal plasma (1:10) is provided for reference. (+) denotes anodal migration. First dimension electrophoresis 30 min, 20 V/cm; second dimension 3 h, 12 V/cm, and antiserum concentration 2.5% v/v.

a more varied precipitin pattern as diffusion rings induced with heparin samples were approximately 10% greater in area than EDTA which in turn were 25–30% larger than the serum samples. The immunoprecipitate obtained with heparin samples was usually less intense and more diffuse than with EDTA, while the serum samples produced an intense characteristic 'double ring' pattern.

Immunochemical differentiation between antithrombin and AT/thrombin complexes. Excess of

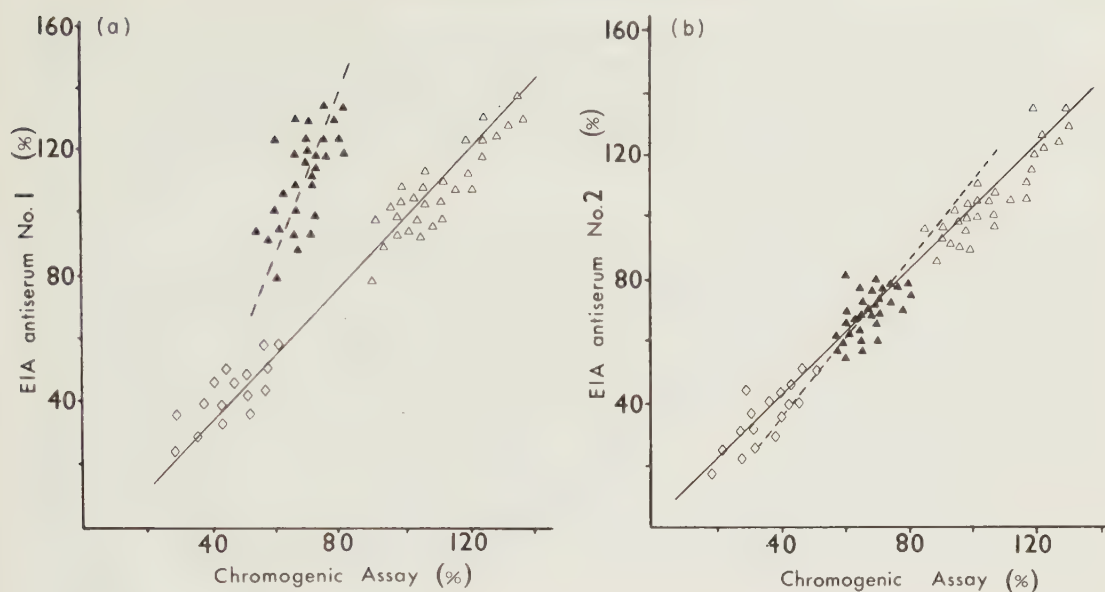


FIG 3. Antithrombin antigen concentration (EIA) compared with antithrombin chromogenic (S2238) activity, 30 normal plasmas (Δ), 30 normal sera ($\blacktriangle$), 16 antithrombin deficiency plasma ($\diamond$). (a) compares the results obtained from antiserum No. 1. The linear regression with normal plasma and antithrombin deficient plasma is $Y=1.115-9.958X$, $R=0.996$; with normal serum, $Y=2.410-5.892X$, $R=0.972$. (b) compares results from antiserum No. 2 linear regression with normal plasma and antithrombin deficient plasma is $Y=0.987+0.402X$, $R=0.97$ with normal serum $Y=1.094-6.175X$, $R=0.945$.

purified AT was added to 500 IU of human thrombin in the resulting mixture analysed immunochemically (Fig 2). An additional inner dense precipitin peak was demonstrated by EIA with antiserum No. 2 and a corresponding reduction in the height of the outer precipitin arc which represented AT. Crossed immunoelectrophoresis of this same mixture, when heparin was incorporated into the gel of the first dimension, separated excess AT from the formed AT/thrombin complex. This newly formed precipitin line was confined to the slower migrating α_2 -AT/thrombin complexes and demonstrated partial immunological identity with the usual AT precipitin reaction.

Comparative analysis of antithrombin. The results obtained by electroimmunoassay and chromogenic assay are compared and summarized in Fig 3. Strong positive correlation was obtained between AT chromogenic activity and electroimmunoassay with both antisera when 30 normal and 16 antithrombin deficiency plasmas were compared ($r=0.96$ and 0.98 respectively). The addition of serum results to the plasma results, however, altered markedly the correlation coefficient with antiserum No. 1 ($r=0.69$). When serum samples alone were compared using antiserum No. 1, the correlation coefficient remained strongly positive ($r=0.97$); however, the slope of the regression line was extremely steep with the intercept some distance away from the origin (Fig 3a).

Addition of thrombin to plasma. The effect on AT levels after increasing amounts of purified human thrombin were added to plasma was variable. Antiserum No. 1 demonstrated little quantitative change regardless of the amount of thrombin added. The electroimmunoassay

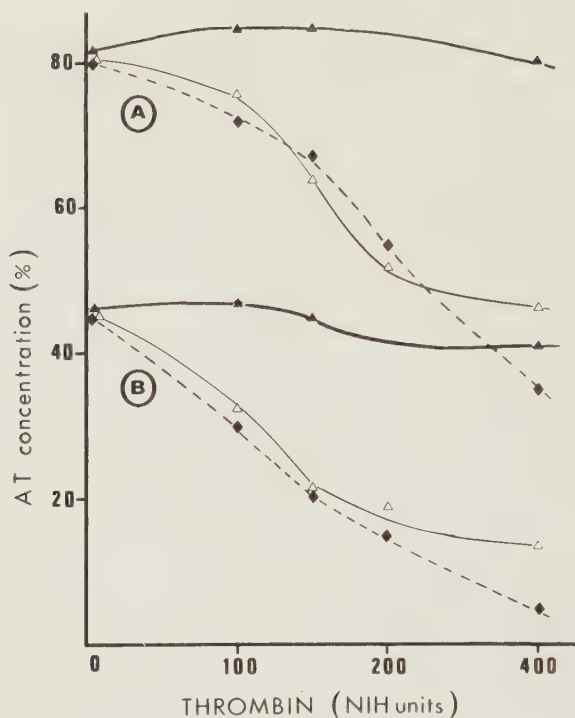


FIG 4. The effect of adding increasing amounts of purified human thrombin on antithrombin levels in two plasma samples. Sample (A) had initially 80% AT activity and sample (B) 45% AT activity. ◆, Chromogenic assay (S2238); ▲, electroimmunoassay results using antiserum No. 1; △, electroimmunoassay results using antiserum No. 2.

results obtained with antiserum No. 2, however, followed more closely the AT chromogenic activity (Fig 4).

DISCUSSION

Should the Measured Levels of Antithrombin III Reflect Biological Activity?

There is a clear correlation between decreased plasma AT activity levels and the incidence of thrombotic disease. Consequently results obtained by any method should reflect the biological activity of AT. There appears to be three major patho-physiological states where clinically significant depressed levels of AT occur: (a) a reduced synthetic rate, e.g. congenital deficiencies (Egeberg, 1965) and liver disease (Damus *et al*, 1965); (b) an increased loss, e.g. glomerular lesions and protein losing enteropathy (Thaler *et al*, 1978); (c) increased consumption, e.g. disseminated intravascular coagulation (Damus *et al*, 1975) and thromboembolism.

These situations and the consequent fate of AT need to be considered when one determines AT levels immunochemically. Decreased antigen levels usually correspond to reduced AT activity levels in the first two categories; therefore both antisera described here can be used equally well. However, when there is increased consumption (category c), inactive AT/protease complexes may be present in addition to depressed AT levels (Sas *et al*, 1977). Therefore

falsely high results will be produced by electroimmunoassay if the antiserum used cannot differentiate between active and inactive AT/protease complexes in the test plasma.

The obvious quantitative disparity demonstrated when plasma and serum were compared directly or when increasing amounts of thrombin were added to plasma (Fig 4) illustrates the limitations of this type of antiserum.

Antithrombin Antisera may Recognize Different Antigen Populations

Conformational changes in proteins can significantly influence antigenic reactivity (Atassi, 1977). Considerable structural changes in AT can be induced by heparin alone (Villanueva & Danishefsky, 1977) and on complexation with proteases (Rosenberg & Damus, 1973). Quantitative and morphological differences by electroimmunoassay with plasma and serum indicated that one antiserum (No. 2) was capable of reacting with additional yet different AT antigenic determinants. The addition of thrombin to purified AT induced the same characteristic 'double peak' immunoprecipitate observed with serum. The newly formed precipitin line was identified as AT antigenic determinants which emerge after conformational changes have been induced by protease/complex formation in addition to those present on the parent AT molecule.

Consequently this antiserum was not only capable of measuring biologically active AT but also able to differentiate it from inactive AT/protease complexes. The electroimmunoassay results therefore correlated much more closely with the chromogenic assay results when the two different populations of AT were present in the test samples. This is highlighted in Fig 4 where increasing amounts of thrombin were added to plasma samples with previously known AT levels.

Disparity between Single Radial Immunodiffusion and Electroimmunoassay

The obvious quantitative differences between plasma and serum samples when tested by SRID and electroimmunoassay using antiserum No. 1 emphasizes the fundamental differences between the two methods. AT/protease complexes which have a higher molecular weight than their native proteins will diffuse more slowly during incubation in SRID. Fagerhol & Abildgaard (1970) have previously discussed these differences showing a maximal difference between plasma and serum of approximately 32% after 18 h incubation. As SRID relies principally on diffusion both antisera can be used equally well and the results obtained should bear a reasonably close relationship to biological activity. Conversely, electroimmunoassay relies on electrophoretic migration (Laurell, 1972). AT and AT/protease complexes demonstrate similar electrophoretic mobility in agarose (unpublished observations). Therefore, unless the AT antiserum can differentiate between the two molecular species of AT during electroimmunoassay the results obtained will reflect the sum concentration of both AT populations as they are equally immunogenic (Fig 4).

Antiserum Production

AT antiserum which recognizes and differentiates between the two antigenic populations on AT has now been produced on four separate occasions. This indicates that this type of antibody specificity is not only reproducible but is perhaps the more normal immunogenic response. To

account for induction of additional antigenic determinants one must therefore assume that human AT complexes *in vivo* with rabbit thrombin during immunization.

An antibody, such as antiserum No. 1, which has limited specificity can be prepared from antiserum No. 2 by the addition of AT/protease complexes.

Choice of Standard and Test Sample

Plasma collected into tri-sodium citrate and EDTA Na₂, which normally has a minimum of AT/protease complexes, should be used as test sample. The AT standard should ideally be defined both functionally and immunochemically, but is unavailable commercially. Therefore fresh pooled normal plasma, stored at -70°C , is the only other alternative. Discrepancies illustrated in Figs 3 and 4 indicate serum or similar product should not be considered.

Immunochemical Methods v. Chromogenic Assays

Newer chromogenic assays for AT exploit heparin's catalytic role to greatly minimize the reaction time (Abildgaard *et al*, 1977). SRID requires 30 h for acceptable results while EIA under these conditions requires 3½.

Most AT deficiency cases have both reduced activity (chromogenic or heparin cofactor) and antigen concentration; however, rare variants have been described. Sas *et al* (1974) have presented cases where AT-heparin cofactor activity was reduced, but antigen concentration was normal. Conversely, a clinically normal individual has been recently described with very similar results (Penner *et al*, 1979). Sas *et al* (1979) and Wolf *et al* (1979) have also described antithrombin deficiency patients which have an intermediate AT-heparin cofactor activity with reduced antigen concentration. Therefore in these very rare deficiency states *both* chromogenic and immunochemical analysis are obligatory plus further AT assays which do not reflect heparin cofactor activity.

ACKNOWLEDGMENTS

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The interaction of heparin with plasma proteins

Demonstration of different binding sites for antithrombin III complexes and antithrombin III

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Affinity chromatography of plasma and serum with the use of heparin conjugated Sepharose has confirmed the existence of two types of protein binding sites. A minor heparin fraction binds free AT III selectively and firmly but not its protease complexes. The complexes bind less firmly to another, much larger heparin fraction together with a select group of the plasma proteins at physiological pH and ionic strength. These included complement proteins (C1q, C2, factor B, properdin, and β 1H), protease inhibitors (inter- α -trypsin inhibitor and C3b inactivator) and cell surface proteins (protein HC and fibronectin) as well as β -lipoproteins. The results demonstrate that affinity chromatography with heparin-Sepharose is extremely useful as an early preparative step in the isolation of these minor plasma components. The results also indicate that the said proteins can be linked to cell surfaces carrying heparinoids in the intercellular space. (J LAB CLIN MED 95:69, 1980.)

Abbreviations: antithrombin III (AT III), acid-citrate-dextrose (ACD), human complex-forming glycoprotein, heterogeneous in charge (protein HC), β -globulin regulating alternate pathway of complement (β 1H)

Heparin is naturally occurring, sulfated, heterogeneous glycosaminoglycan with a range of molecular weights between 5000 and 50,000 daltons.¹⁻³ Since its discovery by McLean in 1916,⁴ evidence has accumulated which demonstrates that heparin functions as a powerful anticoagulant which dramatically enhances the neutralizing effects on thrombin and factors IXa, Xa, and XIIa via the major coagulation protease inhibitor, AT III.^{2, 5}

The major action of heparin has been considered anticoagulant; however, less than one third of the product in all commercial preparations appears to be responsible for exerting anticoagulant activity.^{1, 3} The relationship between the complex structure of heparin and its biological functions is not yet fully defined. In addition to interaction with AT III, other binding properties have been described which implied other biological functions. For example, heparin has the ability to release lipoprotein lipases from tissue sites into the blood.^{6, 7} It can also react with C1q and β -lipoproteins under physiological conditions,^{8, 9} and it binds weakly to fibrinogen.¹⁰

Anticomplementary activity of heparin has been ascribed to either its direct interference with C1q binding to immune complexes^{8, 11} or its inhibition of the reaction between

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C2 and C4b by complexing with Mg^{++} ions.¹² Indirect evidence suggests that heparin potentiates the effect of C1 inactivator and may also contribute to modulation of the amplification pathway of complement via C3 convertase.^{13, 14}

During the process of isolating AT III from plasma by heparin affinity chromatography,^{15, 16} we noted that a number of other plasma proteins were retained by heparin-linked Sepharose. In order to clarify the usefulness of heparin affinity chromatography on plasma fractionation, the proteins bound to and subsequently eluted from heparin-Sepharose were identified with monospecific antibodies.

Material and methods

Samples. Fresh ACD plasma or serum was obtained from healthy blood donors. These samples were subjected to chromatography within 4 hr of collection or divided into suitable aliquots and stored at -70°C until used.

Chromatographic procedures. Four different preparations of heparin-Sepharose were tested during these studies. Heparin (Vitrum Laboratories, Stockholm, Sweden) was coupled to Sepharose 4-B (Pharmacia Fine Chemicals AB, Uppsala, Sweden) according to the method of Miller-Anderson et al.¹⁵ Cyanogen bromide-activated Sepharose 4-B, in which the reactive groups were blocked with 1M ethanolamine, was used as a control.

All chromatographic experiments were carried out at 4°C . A 2.1 by 15 cm column of heparin-Sepharose as well as the control Sepharose 4-B was prepared and equilibrated with the use of 500 ml of 0.05M Tris HCl, 0.01M Na citrate in 0.15M NaCl, pH 7.4. Plasma (50 ml) or serum (65 ml) was applied directly to the column at a flow rate of 20 ml/hr, after which unbound proteins were removed by washing for 18 hr with the starting buffer. Proteins bound to heparin-Sepharose were eluted at a similar flow rate with a linear salt gradient which ranged from 0.15M to 1.5M NaCl in 0.1M Tris HCl, 0.01M Na citrate, pH 7.4. Elution was monitored at 280 nm.

In order to provide near-physiological salt conditions, calcium (2 mM) and magnesium (0.8 mM) ions were added to the starting buffer prior to equilibration of the column for subsequent chromatographic analysis.

Agarose electrophoresis. Agarose electrophoresis¹⁷ was run in 1% (w/v) agarose gel (Marine Colloids, Inc., Rockland, Me.), with a barbital buffer (0.075M, pH 8.6, Ca^{++} 0.002M). Aliquots (15 μl) of the samples eluted were applied directly to the gel and subjected to electrophoresis at 20 V/cm for 45 min. The resulting protein patterns were stained with 0.5% (w/v) Coomassie brilliant blue (BDH Fine Chemicals, Ltd., Poole, England).

Immunochemical analysis. Aliquots of eluate fractions were individually subjected to immunoelectrophoretic analysis¹⁸ against antisera to 45 different human plasma proteins (Table I), most of which were available at this laboratory. Complement proteins, C1r, C1s, C2, properdin, C3b inactivator, and $\beta 1\text{H}$ were identified and quantitated by Dr. A. B. Laurell, Department of Microbiology, University of Lund, Lund, Sweden.

Proteins identified were then subjected to quantitative analysis by electroimmunoassay.¹⁹ The percent recovery of specific proteins was determined from the concentration in the sample applied to the column. The concentration of trace substances such as C-reactive protein, protein HC, and some complement factors not detected by conventional immunoelectrophoresis was determined by the more sensitive electroimmunoassay. Crossed immunoelectrophoresis²⁰ was performed on some proteins to assess whether any changes in heterogeneity had occurred.

Preparation of pure AT III and AT III complexes. AT III was purified from fresh normal human plasma according to the method of Miller-Anderson et al.¹⁵ The final preparation gave a single protein band on gel electrophoresis. Immunoelectrophoresis against an equine anti-whole human serum antibody revealed only one precipitin line which was identified with monospecific rabbit anti-human AT III. All this protein migrated as a prealbumin in the presence of heparin (20 IU/ml of gel).

AT III complexes were recovered from normal human serum by heparin parin affinity chromatography followed by gel filtration over Sephacryl 200 (Pharmacia). Contaminating proteins, i.e., fibronectin, complement C3, and protein HC, were not removed. Crossed immunoelectrophoresis of AT III in the presence of heparin (20 IU/ml of gel) in this fraction did not alter its migration as a fast α_2 -globulin. In addition, the characteristic immunoprecipitation pattern observed established its identity with AT III/protease complexes. Previous analysis of this unique immunoprecipitation reaction

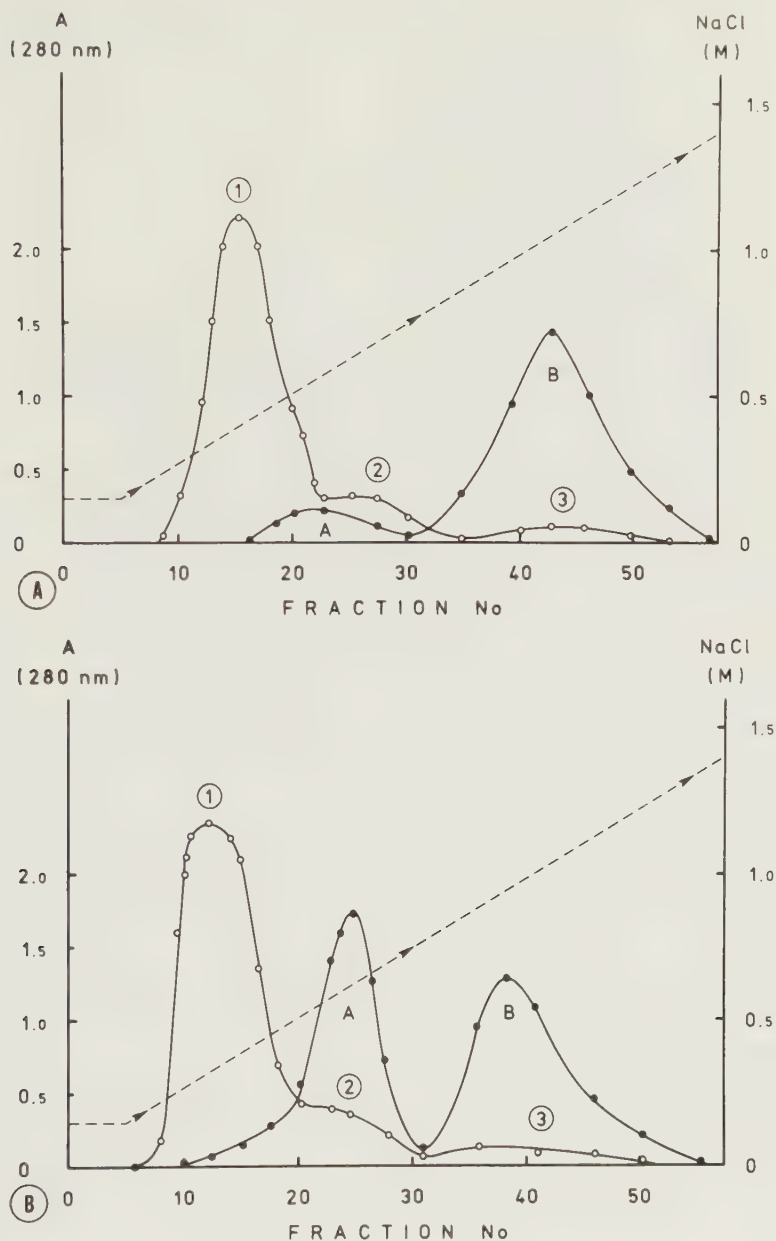


Fig. 1. Characteristic elution patterns after 50 ml of normal plasma (a) or 65 ml of normal serum (b) has been applied to heparin-Sepharose (bed volume 50 ml). All affinity chromatographic experiments were carried out at 4° C, and samples were applied directly at a flow rate of 20 ml/hr. The column was washed free of unbound proteins with a minimum of 5 times the column bed volume, by using the equilibration buffer. Proteins bound to the heparin gel were eluted by a linear salt gradient consisting of 200 ml of 0.01M Tris HCl, 0.01M Na citrate in 0.15M NaCl, pH 7.4, contained in the mixing chamber and 200 ml of 0.01M Tris HCl, 0.01M Na citrate in 1.5M NaCl, pH 7.5 in the reservoir. ○—○, Absorbance of eluted fractions measured at 280 nm and resultant peaks numbered 1, 2, and 3; ●—●, immunoreactive AT III as measured by electroimmunoassay. The two peaks obtained are labeled A and B; ----, calculated linear salt gradient.

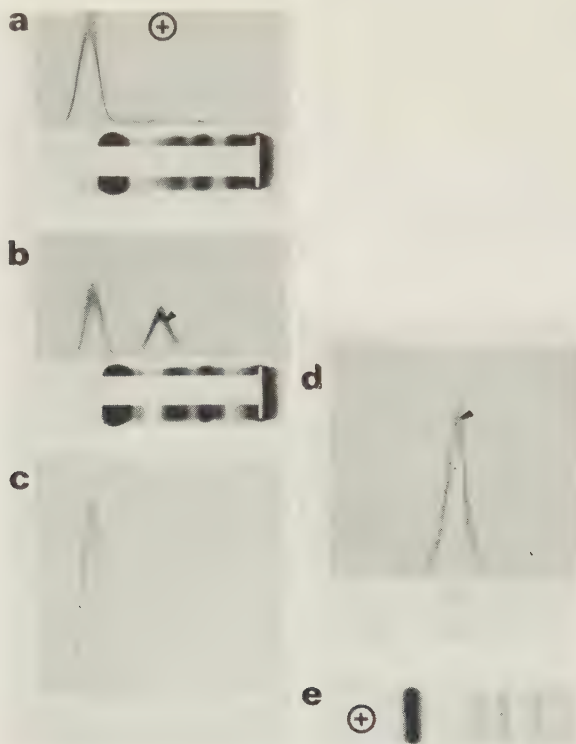


Fig. 2. Crossed immunoelectrophoretic patterns of the various forms of AT III. *a*, Normal EDTA plasma. *b*, Normal serum. *c*, Isolated free AT III. *d*, Isolated AT III/protease complexes. *e*, Electrophoretic pattern of normal plasma diluted 1:6 to compare the migration differences between free AT III and AT III complexes. $\oplus$, Anodal direction; arrows in *b* and *d* indicate the distinct double precipitin lines that are produced by "neo"-antigenic determinants on AT III after its complexing with coagulation proteases.

had shown it to be due to the exposure of "neo"-antigenic determinants on the AT III molecule after its formation of complexes with coagulation proteases.²¹

Fig. 2 demonstrates the electrophoretic and immunochemical differences observed with these fractions.

To elucidate whether free AT III and AT III complexes have different heparin binding sites, purified fractions of these components were reapplied in excess amounts, both together and separately, to heparin-Sepharose. Binding sites on the column were considered saturated only when the purified AT III fractions were detectable in the void volume by electroimmunoassay and confirmed by crossed immunoelectrophoresis.

Results

Heparin affinity chromatography. On the basis of initial experiments performed, 1 ml of plasma or 1.3 ml of serum per milliliter of heparin-Sepharose gave optimal recovery of AT III (90% to 95%). This protein binds more firmly than the other plasma proteins. Less than 5% of AT III was recovered in the void volume under these conditions.

Examples of plasma and serum fractionation experiments are presented in Fig. 1, *a* and *b*. Three protein fractions were invariably resolved: the major one (1) between 0.25M and 0.45M NaCl, a minor but discrete one (2) between 0.45M and 0.60M NaCl, and very small but rather broad one (3) from 0.65M to 1.2M NaCl.

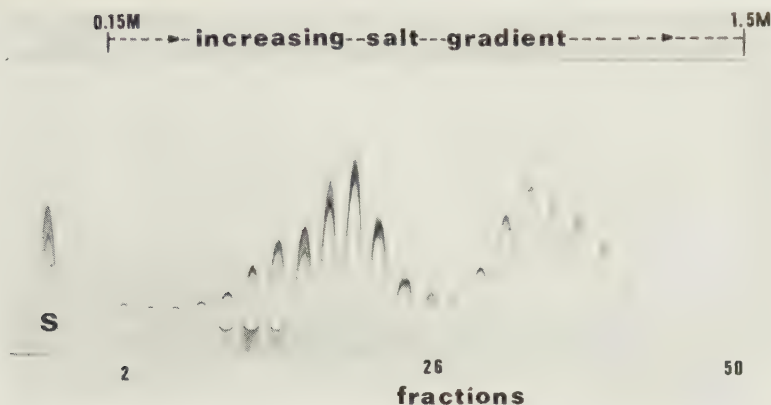


Fig. 3. Immunoreactive AT III of eluted fractions as assayed by electroimmunoassay (compare Fig. 1, *b*) following heparin-affinity chromatography of 65 ml of normal serum. AT III complexes formed during clotting produced distinct immunoprecipitation patterns and were eluted with a salt concentration that ranged between 0.30M and 0.65M NaCl (peak A). "Double" rockets were demonstrated by the complexes with one sharp precipitin line inside a more diffuse rocket outline. Noncomplexes AT III (peak B), which has a higher affinity for heparin, was eluted at a salt concentration greater than 0.65M NaCl, and the rockets obtained produced a more diffuse, single precipitin line. S, 1:5 dilution of the serum sample employed for affinity chromatography.

When plasma (Fig. 1, *a*) was chromatographed, fractions 1 and 2 contained less than 10% of immunoreactive AT III (peak A) eluted from the column. The third one (3) contained approximately 90% of AT III (peak B) recovered in the eluate. Crossed immunoelectrophoresis of a concentrated aliquot of the fractions between 0.7M and 1.2M NaCl revealed that AT III thus obtained was electrophoretically homogeneous. In the presence of 20 U/ml heparin in the electrophoretic gel, AT III migrated as a single band in the prealbumin region (Fig. 2).

A slightly higher peak 2 was obtained by chromatography of serum (Fig. 1, *b*). The AT III immunoprecipitation pattern obtained here, however, revealed a major peak (A) which occurred between 0.4M and 0.65M and could be distinguished visually by its distinct immunoprecipitation characteristics from the one eluted at 0.65M to 1.2M NaCl (Fig. 3). Crossed immunoelectrophoresis with a concentrated aliquot of the earlier fractions revealed that this AT III migrated as a fast α_2 -globulin when heparin (20 IU/ml) was incorporated in the gel and also demonstrated the characteristic double precipitin lines (Fig. 2).

When excess purified AT III (6 times the normal sample load) was applied to the column, only 15% was bound (Fig. 4, *a*). The pattern of elution was similar to that observed with normal plasma; that is, most of AT III was eluted between 0.65M and 1.2M NaCl.

AT III complexes, when applied to heparin-Sepharose in the absence of free AT III, demonstrated the same elution pattern as observed with the complexes in whole serum; that is, they were eluted between 0.4M and 0.65M NaCl only (Fig. 4, *b*). An excess of AT III complexes, in the absence of free AT III, had a similar elution profile and immunoprecipitation pattern as observed with serum; that is, elution occurred between 0.4M and 0.65M NaCl. They also induced the characteristic double precipitin line indicative of AT III complexes. These complexes were not displaced from heparin-Sepharose by subsequent, additional application of excess loads of free AT III, as evidenced by the appear-

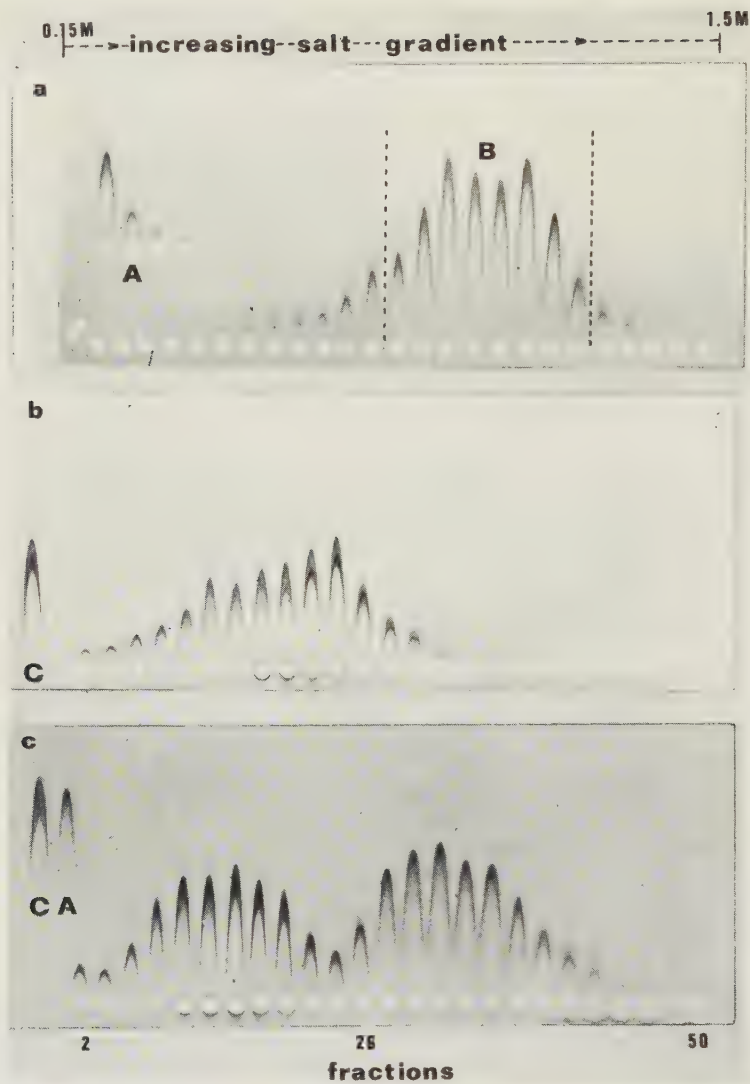


Fig. 4. Immunoprecipitin pattern obtained by electroimmunoassay from eluted fractions. *a*, After excess purified AT III only has been subjected to heparin affinity chromatography. Virtually all AT III bound to heparin-Sepharose has been eluted by a high salt concentration, i.e., between 0.7M and 1.2M NaCl (area B). Rockets labeled A are standard dilutions of purified AT III. ----, Linear gradient. *b*, After excess AT III complexes *only* have been applied to heparin-Sepharose, demonstrating that AT III complexes are still eluted at a much lower salt concentration than free AT III, i.e., between 0.30M and 0.60M NaCl. Rocket labeled C is a standard dilution of AT III complexes. *c*, After excess AT III complexes have been loaded onto the heparin column, washed with the equilibration buffer, and immediately followed by application of excess purified AT III to the same column. Rockets labeled C and A are standard dilutions of AT III complexes and purified AT III, respectively.

ance of free AT III only in the void volume. The resulting elution and immunoprecipitin patterns were similar to those observed when whole serum is applied to heparin-Sepharose; the complexes were eluted between 0.2M and 0.65M NaCl, whereas the purified free AT III eluted between 0.65M and 1.2M NaCl (Fig. 4, c).

Affinity chromatography with control Sepharose 4-B. The chromatographic

Table I. Plasma proteins tested for, and identified as capable of, binding to heparin-Sepharose

Proteins	% recovery*	Proteins	% recovery*
Transport:		Coagulation:	
Prealbumin	—	Prothrombin	—
Albumin	—	Fibrinogen	—
α -Lipoprotein	—	Factor XIII	—
Transcortin	—	Plasminogen	—
Gc globulin	5-10	Factor VIII related antigen	—
Ceruloplasmin	—		
Thyroxine-binding globulin	—	Cell surface:	
β -Lipoprotein	85-95	Protein HC	55-65
Sex hormone binding globulin	—	Fibronectin	65-80
Ferritin	—	β_2 -Microglobulin	—
Transferrin	—	Complement:	
Hemopexin	—	Properdin	60-70
Immunoglobulins:	—	C1q	75-90
IgA	—	C1r	10-25
IgD	—	C1s	<10
IgM	—	Factor B	45-55
IgG	—	β 1H	70-80
Protease inhibitors:		C2	65-70
α -1-antitrypsin	—	C3	10-15
α -1-antichymotrypsin	—	C4	<10
Inter- α -trypsin inhibitor	65-70	Others:	
α_2 -Macroglobulin	—	α -1-acid glycoprotein	—
α_2 -Antiplasmin	—	Acid- α_2 -glycoprotein	—
Antithrombin III	85-95	β_2 -glycoprotein 1	65-70
C1 Inactivator	—	C-reactive protein	85
C3b Inactivator	50-60		

Gc globulin = group-specific component globulin.

*Entry indicates protein identified immunochemically and recovery rate determined by electroimmunoassay from the elution fractions compared directly with the plasma or serum samples prior to heparin affinity chromatography.

procedure followed was identical to that used with heparin-Sepharose. A rather narrow protein peak with an optical density at 280 nm of less than 0.2 was eluted from this column. The fractions obtained were pooled, concentrated, and then subjected to gel electrophoresis and immunochemical analysis. Six plasma proteins were identified: the complement proteins C1q, C2, and C3, properdin, factor B, and fibronectin. However, less than 15% of all six proteins applied to the column was bound to the gel.

Immunochemical identification of proteins. Seventeen plasma proteins that bound to heparin-Sepharose at physiological ionic strength and pH were identified. Although these proteins differ in their capacity to bind heparin, at least 50% of 12 of these proteins were bound to the column. Table I indicates which ones were identified and the percentage of each recovered from the eluate.

Crossed immunoelectrophoresis of eluate fractions rich in protein HC indicated marked heterogeneity. HC/IgA complexes remained intact and were similar to those obtained from normal plasma.²²

Agarose electrophoresis. Agarose gel electrophoresis of the eluted fractions revealed the presence of a series of protein bands that varied in electrophoretic mobility from the mid-gamma zone through to the inter-alpha zone. Almost all these proteins were

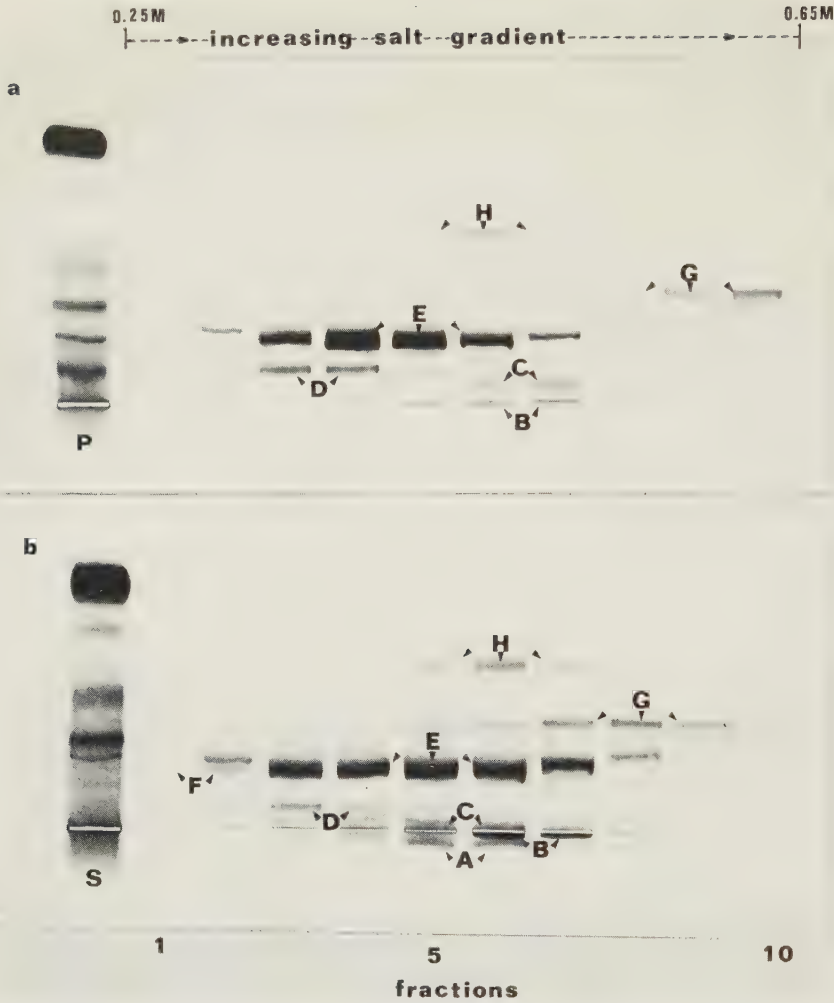


Fig. 5. Agarose electrophoretic patterns of plasma protein fractions eluted from heparin-Sepharose with a salt gradient ranging between 0.25M and 0.65M NaCl. The fractions are numbered 1 to 10, and the major individual proteins identified immunochemically are labeled A to H according to their electrophoretic mobility. *a*, Pattern obtained after 50 ml of plasma had been subjected to heparin affinity chromatography. *P*, 1:5 dilution of the plasma sample employed for chromatography. *b*, Pattern is obtained after 65 ml of serum (in the presence of Ca^{++} and Mg^{++} ions) had been applied to heparin-Sepharose. *S*, 1:5 dilution of the serum sample employed for chromatography. *A*, Properdin, observed as a discrete cathodal protein band in fractions 5 to 8 of *b*. Its separation and concentration are enhanced in the presence of Ca^{++} (2 mM) and Mg^{++} (0.08 mM) ions; *B*, C1q, interacting with the agarose gel and migrating immediately cathodal of the application slit in fractions 6 to 9 of both *a* and *b*; *C*, β_2 -glycoprotein 1, recognized on the anodal side, close to the application slits in fractions 7 to 10 in *a* and fractions 5 to 8 in *b*; *D*, factor B of the alternate pathway of complement, observed in the anodal gamma zone and eluted early on in the gradient; *E*, β -lipoproteins (LDL), seen as the dominating typical, irregular, dense band spanning most of these fractions and masking C4 and C3 proteins, which are also eluted in the early and middle portions of the fractions (the appearance of heterogeneity in the different fractions suggests a subfractionation of LDL); *F*, C2 of the complement proteins, observed as a β -globulin in the first two elution fractions of *b* only (β -lipoprotein also masks the rest of the C2 eluted in the following fractions); *G*, fibronectin in the α_2 - β interzone, eluted as a distinct band in the latter two thirds of fractions; *H*, Inter- α -trypsin inhibitor, recognized as the major protein detected in the fast α_2 -region.

eluted with the first, major component between 0.25M and 0.45M NaCl in contrast to immunoreactive AT III (B) which was the only protein eluted at a concentration of NaCl greater than 0.65M.

Fig. 5 is representative of agarose electrophoretic patterns obtained with the plasma protein fractions eluting from heparin-Sepharose between 0.25M and 0.65M NaCl. Fig. 5, *a*, is that obtained when plasma was applied to the column. Fig. 5, *b*, represents the protein patterns obtained from serum applied to the heparin column in the presence of Ca^{++} and Mg^{++} ions. The major proteins identified immunochemically are labeled according to their electrophoretic mobility, commencing from the most cathodal one.

Discussion

Presence of two types of binding sites in heparin. Heparin has recently been subfractionated according to its affinity for AT III.^{2, 23} Material that has a high affinity for AT III also has high anticoagulant activity, in contrast to low-affinity heparin which has little or no anticoagulant activity.^{2, 23} No attempt was made to separate heparin in this way prior to linking it with Sepharose. Therefore our columns contained unknown mixtures of high- and low-affinity heparins. Previous studies^{24, 25} have demonstrated that AT III which has a high affinity for heparin (our peak B) occurs in free form and acquires markedly increased migration when heparin is incorporated into the agarose prior to electrophoresis. AT III which has complexed with coagulation proteins such as thrombin (our peak A) during the clotting process is much less affected.^{24, 25} AT III in complexed form has a much lower affinity for heparin because its binding sites are masked by complex formation. The ability of heparin-Sepharose to retain more immunoreactive AT III from serum (1.3 times) compared to plasma (1 time) reinforces this concept, for at least 25% of AT III in serum occurs in the form of complexes that do not compete with free AT III for the high-affinity heparin. Conversely, free AT III seems not to compete with other proteins for low-affinity heparin. Loading of an excess of purified AT III onto the column gave no appreciable increase in the amount of AT III bound to heparin (Fig. 4, *a*). The fact that AT III bound to heparin required a higher concentration of NaCl for elution indicates that the heparin-AT III interaction is unique.

The pattern of elution when an excess of AT III complexes was reapplied to the column in the absence of free AT III still demonstrated elution at low salt concentration, i.e., 0.65M or less (Fig. 4, *b*). That these complexes were not displaced from heparin-Sepharose when excess purified AT III was concurrently applied indicates that there are different heparin binding sites, one for free AT III, which has a high affinity but low capacity and requires a high salt concentration for elution, and the other for AT III complexes, which have a low affinity but much higher capacity and elute at a lower salt concentration. These results rather suggest that heparin reacts with the proteins complexed to AT III, since thrombin and factors IXa, Xa, and XIa, which complex with AT III during coagulation, are also capable of binding directly to heparin.²⁶⁻²⁸ The utilization of an antiserum which is capable of distinguishing antigenic differences between free AT III and AT III complexes in terms of a characteristic immunoprecipitin pattern has allowed us to demonstrate different binding sites for these two molecular species of heparin without the need to separate them.

Evidence that a very small proportion of heparin is involved in AT III binding has been provided by Thaler and Schmer,¹⁶ who demonstrated that 3% of heparin bound to agarose was active. Recently Lindahl et al.²⁹ have isolated the specific AT III binding site on heparin as a tetradecasaccharide unit which corresponded to 2% to 3% of high-activity heparin. Bengtsson et al.³⁰ demonstrated that the interaction of heparin with lipoprotein

lipase is different from its interaction with AT III. Niewiarowski et al.³¹ have also shown that platelet anti-heparin proteins and AT III interact with different binding sites on heparin.

Heparin-bound Sepharose appears to be primarily concerned with the binding of a select group of plasma proteins at physiological salt levels, since less than 10% of it interacts directly with AT III.

Type and specificity of protein linkage. The marked variation in electrostatic charge and molecular size of the proteins that bind to heparin-Sepharose indicates that stereospecific interactions in addition to ionic effects are responsible for binding. Divalent cations Ca^{++} and Mg^{++} were not required. However in the presence of Ca^{++} and Mg^{++} , properdin eluted in a much narrower salt range and could be visualized directly as a discrete band on electrophoresis. In contrast, fibronectin was eluted over a much wider salt gradient.

With the exception of β -lipoproteins, none of the transport proteins or immunoglobulins constituting major plasma fractions binds to heparin-Sepharose. Those which are bound can be divided into four functionally related categories; coagulation proteins,^{27, 32} complement proteins, protease inhibitors, and cell surface proteins (see Table I for details). The biologic function of β_2 -glycoprotein 1 has yet to be defined.

Interactions and extracellular functions of heparin. Heparin is well known for its anti-inflammatory capabilities as well as its anticoagulant role and has been shown to inhibit delayed hypersensitivity reactions.³³ This suggests that heparin or heparin-like substances may be directly involved in modulating important biological processes within the extracellular fluids. The recovery of surface-linked proteins from heparin-Sepharose suggests that biological competition may occur between these proteins for the low-affinity (but high-capacity) binding sites of heparin in tissues. The heterogeneous β -lipoproteins are of special interest because they occur at much higher concentrations in plasma than in extracellular fluids. In the latter system they may compete less for the available heparin binding sites. The remarkable enrichment of certain complement proteins obtained by heparin chromatography may be indicative of an ordered process in which heparin can interact directly with them and thereby exert some control over the related processes. Rent et al.¹³ described an indirect potentiating effect of heparin on C1 esterase inhibitor analogous to that of AT III. Our binding studies using purified C1 as well as normal serum indicate that the C1 macromolecule is directly inactivated by heparin, producing C1q and C1r/C1s complexes in proenzyme form. This inactivation is not influenced by C1 esterase inhibitor.³⁴ Sulfated polysaccharides have previously been shown to activate the alternative pathway of complement.³⁵ Direct binding of properdin, factor B, C3b inhibitor, and β 1H to heparin-Sepharose suggests that heparin may not only activate these steps in the sequence of complement events but may also be capable of effecting modulation at its central point, i.e., C3b or C3 convertase, which is necessary for optimal reactive cell lysis.^{14, 36}

Cellular glycosaminoglycans have been implicated in binding fibronectin to cell surfaces. Controversy exists as to whether heparin binds preferentially to fibronectin, to fibrinogen, or both.^{37, 38} Stathakis and Mosesson¹⁰ have recently demonstrated that heparin binds preferentially to fibronectin and weakly to fibrinogen. Our studies however show that fibrinogen does not bind heparin. This difference is probably related to the higher salt concentrations employed in equilibrating our affinity columns. Under our conditions most of the fibronectin (80%) applied to the column was bound by heparin.

The low-affinity, high-capacity sites on heparin involved in selective plasma protein binding could be exploited as an important chromatographic step for the isolation of many

of these proteins that occur normally in low concentration (Table I). This affinity for heparin-Sepharose may well mimic the biological tissue interaction of certain plasma proteins with sulfated glycosaminoglycans.

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A SIMPLE TWO-STEP PROCEDURE FOR THE ISOLATION OF ANTITHROMBIN III FROM BIOLOGICAL FLUIDS

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ABSTRACT

A simple procedure for the isolation of antithrombin (AT) from different biological fluids is described. This method utilizes high molecular weight dextran sulphate in the presence of calcium ions as a first preliminary step to remove most other heparin binding proteins prior to heparin-affinity chromatography. Such a step does not affect free AT from binding heparin but rather allows a much improved yield of AT to be obtained with higher heparin-cofactor activity. By utilizing biological samples with differing AT molecular species, the results obtained, illustrate not only is dextran sulphate capable of removing proteases most likely to complex with AT during heparin affinity chromatography but it can effectively remove preformed contaminating AT/protease complexes as well.

INTRODUCTION

The interaction between antithrombin III (AT) and heparin appears highly specific and induces a rate enhancing effect on the inhibition of activated coagulation proteases- thrombin, factors IX and X (1,2). Furthermore heparin fractions which have highest affinity for AT have greatest anticoagulant activity (2,3). This interaction has led to the use of heparin-affinity chromatography as an important first step in the isolation and recovery of good yields of AT (4,5,6). As the product is rather heterogeneous and minor contaminants remain, further steps are necessary to provide material of acceptable purity, which reduce final recovery and hamper large scale preparation. The specific AT binding site represents 2-3 % of high affinity heparin (7), a fraction which corresponds to less than one third of most commercial heparin preparations (8). Consequently a very small portion of the total heparin conjugated to agarose is capable of binding AT, (i.e. high affinity but low capacity). The major fraction of immobilized heparin (i.e. low affinity but high capacity), binds a group of specific plasma proteins other than AT (9). Such proteins significantly contaminate AT during its elution. Coagulation

Key words: Antithrombin • Dextran Sulphate • Heparin-affinity chromatography

proteases, factors IX and X, bind directly to heparin as well, and heparin-affinity chromatography may potentiate their activation with the formation of AT/protease complexes during chromatography.

In order to facilitate optimal binding to, and maximize recovery of, homogeneous active AT from heparin-Sepharose a simple early step is described for removing most proteins capable of linking "low affinity" heparin sites. Dextran sulphate in the presence of divalent cations has been used extensively for precipitating out low density lipoproteins (10,11), quantitatively, the dominant heparin interacting plasma protein. This highly sulphated polysaccharide has a strong affinity for many other heparin-binding plasma proteins e.g. Clq, fibronectin, and coagulation proteases, thrombin, factors IX, X, and protein C (12,13,14,15).

AT isolated by the 2 step procedure detailed here is homogeneous, has high heparin co-factor activity and is obtained in very high yields.

MATERIALS AND METHODS

Preparation of Chromatography Samples

Fresh normal citrated plasma, pooled serum, and ascites fluid (from patients with liver metastases) were collected, and stored in aliquots at -20°C until required. 1 ml of 10 % aqueous dextran sulphate mol.wt. 500,000, (Pharmacia) plus 1 ml of 5 M aqueous CaCl_2 were added to each 100 ml sample. The mixture was stirred continuously for 15 min at 20°C , centrifuged for 20 min at 3000 x g and the supernatant retained.

Heparin-Sepharose Chromatography

100 ml samples of plasma and serum or 200 ml ascites fluid (with and without dextran sulphate precipitation) were applied directly to a column of heparin-Sepharose (9).

Gel Electrophoresis and Immunochemical Analysis

Agarose and polyacrylamide gel electrophoresis were performed as previously described (16,17). Crossed immunoelectrophoresis (18) was performed using 20 IU heparin (Vitrum Laboratories) per ml of gel in the first dimension to differentiate and assess the relative concentrations of AT and non-active AT/protease complexes contained in the chromatographic samples. Electroimmunoassay (EIA) (19) was used to assess and localize eluted immunoreactive AT from the chromatograms. Rabbit anti-AT, capable of differentiating native AT from AT/protease complexes by a specific immunoprecipitation reaction pattern (20) was used throughout these studies.

Heparin Co-factor Activity

AT functional activity was measured by its ability to inhibit the amidolytic activity of thrombin in the presence of excess heparin (21).

RESULTS

Effect of Dextran Sulphate on Antithrombin III

The effect dextran sulphate precipitation had on AT contained in the three types of samples prior to chromatography was assessed by crossed immunoelectrophoresis.

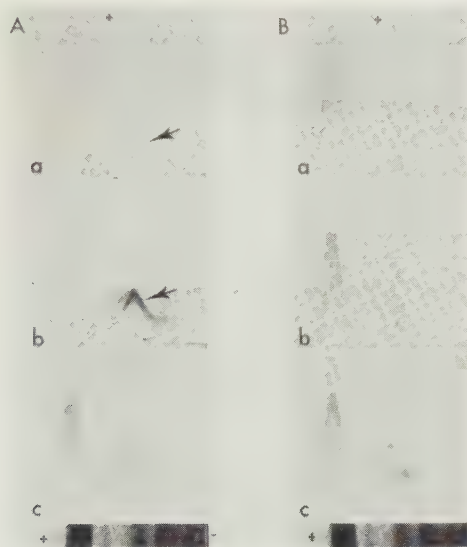


Fig. 1

Crossed immunoelectrophoresis of AT before A), and following dextran sulphate precipitation, B). a) citrated plasma b) pooled serum and c) ascites fluid. Arrows denote distinctive immunoprecipitate obtained with AT/protease complexes.

All samples demonstrated one major AT immunoreactive peak in the prealbumin position with two minor peaks in the slower α globulin region prior to dextran sulphate precipitation. These peaks occurred in varying proportions and revealed different precipitation morphology. The smaller peaks in plasma (Fig. 1Aa) constituted $\sim 5\%$ of the total AT, while in serum (Fig. 1Ab) represented $\sim 35\%$ and in ascites fluid $\sim 45\%$ of the total AT (Fig. 1Ac). Dextran sulphate precipitation of the same samples (Fig. 1Ba, b, c, respectively) resulted in the removal of the slowest migrating AT species from plasma and serum but not from ascites fluid.

Heparin-affinity Chromatography before and after Dextran Sulphate Precipitation

The elution patterns following heparin chromatography of fresh plasma are provided in Fig. 2.

TABLE 1
Recovery of Antithrombin III

Starting Material		Heparin-Affinity Chromatography				Dextran Sulphate + Heparin Affinity			
Sample	AT conc (mg/Ag) ^a	Heparin Cofactor ^b (U/mg AT)	AT conc (mg/Ag)	Reco- very (%)	Heparin Cofactor (U/mg pro- tein)	Reco- very (%)	AT conc (mg/Ag)	Reco- very (%)	Heparin Cofactor (U/mg pro- tein)
Plasma	28.0	4.4	20.2	72	3.9	64	26.4	94	4.3
Serum	16.0	4.0	12.5	78	3.4	66	14.2	88	4.0
Ascites Fluid	47.6	2.5	20.1	42	3.1	52	26.1	55	4.2

The figures provided represent mean values of duplicate runs.

^aDetermined by EIA and compared to a standard of previously isolated AT for which the concentration was calculated by extinction at 280 nm. ^bDetermined by release of p-nitroaniline from S2238 using the same AT standard as in a).

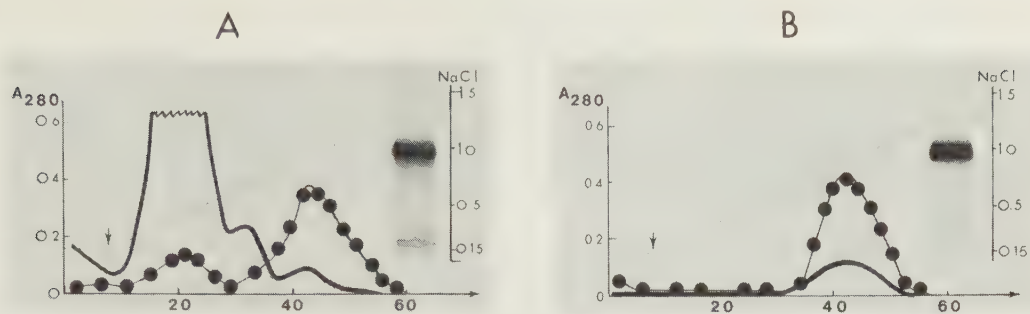


Fig. 2

Heparin-affinity chromatography of 100 ml citrated plasma before A) and following dextran sulphate precipitation B). Arrows indicate commencement of elution developed with 300 ml linear salt gradient. Solid line denotes protein concentration at 280 nm, ●—● antithrombin antigen measurement. Agarose gel electrophoresis inserts illustrate protein patterns obtained after respective elution fractions were pooled and concentrated to OD_{280} of 3.0.

The difference in results illustrates the effectiveness of dextran sulphate to remove virtually all proteins capable of binding to and being eluted from heparin-Sepharose at 0.15 to 0.6 M NaCl. The agarose gel electrophoresis inserts provide the protein patterns obtained from fractions eluted between 0.7 and 1.5 M NaCl from each chromatogram, pooled and concentrated to an OD_{280} of 3.0. Table 1 summarizes and compares the recovery of AT from the various samples with and without dextran sulphate precipitation. The purity of isolated AT obtained by the two step procedure was checked by agarose gel electrophoresis with and without heparin in the gel (Fig. 3A), gradient slab PAGE (Fig. 3B) and crossed immunoelectrophoresis against rabbit anti whole human serum. AT appeared as a monoband protein, with a MW approximately 65,000 and an estimated purity of more than 95 %.

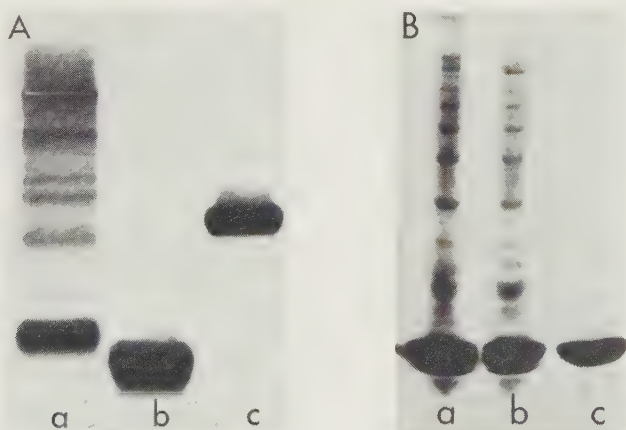


Fig. 3

A) Agarose gel electrophoresis of purified AT with and without the influence of heparin. a) Normal plasma 1:10, b) 30 μ g isolated protein run in the presence of 50 IU heparin c) same as b) but without heparin. B) Gradient slab PAGE (4-30 %). 5 μ l ascites fluid a) before b) following dextran sulphate precipitation c) 30 μ g protein obtained from concentrated elution pool 0.7-1.5 M NaCl.

DISCUSSION

The different biological fluids subjected to chromatography disclosed the existence of at least three molecular species of AT with regard to their affinity for heparin. Native AT which has a high affinity for heparin (all samples), AT/protease complexes which have reduced affinity for heparin (plasma and serum), and AT which has no heparin affinity (ascites fluid).

Dextran sulphate effectively removes preformed AT/protease complexes apparently without interfering with AT/heparin cofactor activity. Preliminary dextran sulphate treatment of samples allows recovery of AT in higher yields and with greater purity as activation of proteases during chromatography is also avoided by prior removal of potentially reactive zymogens. Such a step ensures a more stable preparation of AT during storage. The elution chromatogram (Fig. 2a) illustrates that such zymogen activation does take place during chromatography producing a greater yield of AT/protease complexes than was found in the original sample (20 % compared to 5 %).

That dextran sulphate induced precipitation removes preformed AT/protease complexes from plasma and serum was substantiated by demonstrating their content in the solubilized precipitate. Additional affinity chromatography experiments utilizing dextran sulphate derivatized Sepharose also demonstrated the selective removal of AT/protease complexes from the same samples.

Samples not subjected to dextran sulphate precipitation all gave reduced recovery of total AT protein and reduced heparin cofactor activity on affinity chromatography. This suggests that saturation of the affinity resin with heparin binding proteins other than AT (eg. low density lipoproteins) can physically mask specific heparin binding sites for AT. Consequently the maximum AT load that can be retained on the resin is hampered and AT isolated is significantly contaminated by these proteins during its elution.

Dextran sulphate appears to share with low affinity-high capacity heparin fractions the property of binding certain proteins and AT/protease complexes (9) without interfering with free AT. These results also allow some conclusions to be drawn with regard to specificity and structural requirements on heparin for proteins other than AT. Dextran sulphate and heparin are both highly sulphated polysaccharides suggesting sulphate groups play a role in heparin's major plasma protein binding capacity whereas sulphate groups alone are not responsible for the specific heparin/AT interaction (7). Initial experiments using hyaluronic acid, a non sulphated glycosaminoglycan, failed to induce the same precipitation effects, indicating structural specificity beyond the polyanionic properties of heparin are involved. The effective removal of AT/protease complexes from plasma and serum by dextran sulphate without affecting native AT provides evidence that AT acquires new reactive sites following complex formation with proteases. This results in stronger binding to low affinity heparin and dextran sulphate than with native AT. The apparent loss of binding to high affinity heparin suggests that steric hindrance or loss of this part of the AT molecule has occurred.

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PRACTICAL REQUIREMENTS FOR IMMUNOCHEMICAL ANALYSIS OF ANTITHROMBIN III IN AGAROSE GELS

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Key words: Antithrombin III; immunochemical analysis; heparin; agarose

SUMMARY

This report emphasizes the importance charged groups affixed to the agarose gel matrix have on immunoprecipitation of antithrombin III (AT) and of its protease complexes. Plasma and serum samples were analyzed for antithrombin III by electroimmunoassay and single radial immunodiffusion in different types of agarose. The results were compared with the amount of functionally active AT contained in the samples. A high correlation between the results was obtained provided the following criteria were fulfilled: 1) Agarose with sulphate content of 0.2 % or more was used; 2) The antiserum should contain antibodies directed to AT plus antigenic determinant(s) that emerges during the AT-protease interaction; 3) A gel buffer of about 75 mmol/L was used.

Heparin added to the test system competed with sulphate groups affixed to the agarose gel matrix and inhibited immunoprecipitation of AT.

INTRODUCTION

A strong correlation between low plasma antithrombin III (AT) functional activity, i.e. its capacity to inactivate thrombin, and a high incidence in thrombosis has been demonstrated for both inherited AT deficiency and disseminated intravascular coagulation (DIC), (1-3). Therefore values reflecting the functional activity of plasma AT are clinically of prime importance. Immunological procedures for assessing AT in plasma have been reported with both strong and poor correlation between immunoreactive and functional AT levels (4-6). Immunochemical procedures estimate AT content based on antigenic reactivity irrespective of AT functional activity. Consequently, immunochemical results may be misleading where mixtures of native AT and appreciable amounts of AT/protease complexes occur in the sample, e.g. in DIC (7). We have recently demonstrated that a high correlation can be obtained between immunoreactive plasma AT and its functional activity measured by its capacity to inhibit thrombin cleavage of a specific chromogenic substrate. These results apparently depended upon an antiserum that recognized and distinguished separate antigenic determinants on native AT and AT/protease complexes (8).

This report emphasizes the influence charged groups affixed to the agarose gel matrix may have on immunoprecipitation of AT and AT/protease complexes. Decreasing the ionic concentration of the electrophoresis buffer or adding heparin to the samples or test systems were shown to have unfavourable effects on immunochemical quantitation of AT.

MATERIALS AND METHODS

Test Samples: EDTA plasma and corresponding sera from 15 blood donors were collected and stored at -70°C in small aliquots until tested.

Standard Plasma: Fresh human plasma from 10 assumed healthy blood donors were collected into Na_2EDTA , pooled and stored at -70°C as small aliquots. This plasma pool was given an arbitrary figure of 100 % for both immunochemical and functional activity assays.

Purified Antithrombin: Antithrombin was isolated as described earlier (9). Its protein concentration was estimated by accepting a specific absorbance (1 %, 1 cm) of 6.5 at 280 nm.

Agaroses: Four batches of agaroses differing in sulphate content were used.

a) Agarose (B) with electroendosmosis (EEO) and relative migration rate (mr) of -0.25 and sulphate content of 0.30 % (lot No CH813) was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

b) Agarose (Seakem) with EEO mr of -0.16 and sulphate of 0.20 % (lot No 59030) was purchased from Marine Colloids, Rockland, USA.

c) Agarose (HSC) with EEO mr of -0.01 and a sulphate content of 0.01 % (lot No 0443) was purchased from Litex, Copenhagen, Denmark.

d) Agarose (HSIF) with EEO mr of 0.00 and all sulphate groups "blocked" (lot No 507) was purchased also from Litex.

Antiserum: Rabbit anti-antithrombin III was obtained after repeated subcutaneous injections of 0.2 mg of purified AT emulsified in complete Freund's adjuvant into rabbits. This antiserum demonstrated a single line of immunological identity by double immunodiffusion with purified AT, normal plasma and serum and was shown previously to be capable of differentiating native AT from AT/protease complexes by electroimmunoassay (EIA), (8).

Buffer: Sodium barbitone/barbituric acid (75 mmol/L), pH 8.6, was used throughout unless stated otherwise.

Heparin: Heparin from porcine intestinal mucosa with stated anti-coagulant activity of 5000 IU/ml was purchased from Vitrum Laboratories, Stockholm, Sweden.

Immunochemical Procedures: Single Radial Immunodiffusion (SRID) was performed at 20°C with a diffusion time of 30 hours (10). Electroimmunoassay was performed at 12 V/cm for 4 hours (11).

Functional Antithrombin III Activity: Antithrombin functional activity was measured by its ability to inhibit amidolytic activity of thrombin in the presence of excess heparin, (12). The reagents were kindly supplied in complete kit form (COATEST^R) by Kabi Diagnostics, Stockholm, Sweden.

Calculations: Correlations were calculated by the method of the least squares.

RESULTS

The effect of different agarose gels on immunoprecipitation of AT using purified AT is summarized in Fig 1. Electroimmunoassay in desulphated gels demonstrated higher and more diffuse precipitin loops than in sulphated gels. This contrasted with SRID precipitin areas produced in desulphated gels that were smaller than those formed in sulphated gels.

Serum samples invariably produced a characteristic precipitin pattern by EIA with two precipitin loops, one inside the other (see Figs 4 and 5). The inner dense line corresponds to AT/protease complexes while the outer, weaker line represents native AT (8).

Antithrombin concentration obtained by immunochemical and activity analysis of plasma and serum samples in various types of gels that gave high and low correlation respectively are compared as scatter plots in

Fig 2. Correlation coefficient and lines of regression for the results, obtained after immunochemical analyses run in the four types of agarose gels, are provided in Table 1. Strong positive correlation was obtained between immunochemical and functional assays when sulphate gels were used, whereas poor correlation was obtained using desulphated gels.

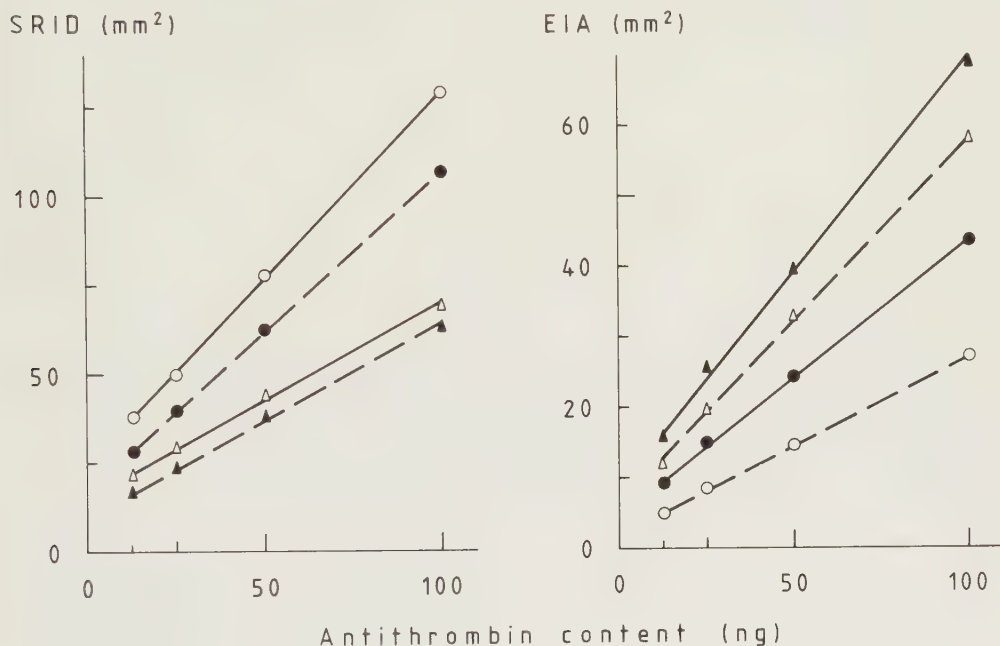


Fig 1. The effect of different agaroses on the immunoprecipitation areas obtained by SRID and EIA using the same concentrations of antiserum and isolated AT. Agarose type B ○-○, Agarose ME ●-●, Agarose HSC △-△, and Agarose HSIF ▲-▲.

The EIA precipitin loop area and the precipitin area for SRID increased when heparin was added to type ME agarose (25 IU/ml of gel), and is illustrated in Fig 3. Antithrombin precipitin loops were rather diffuse when formed in the presence of heparin and made the final precipitates difficult to measure. The inner precipitin loop identifying

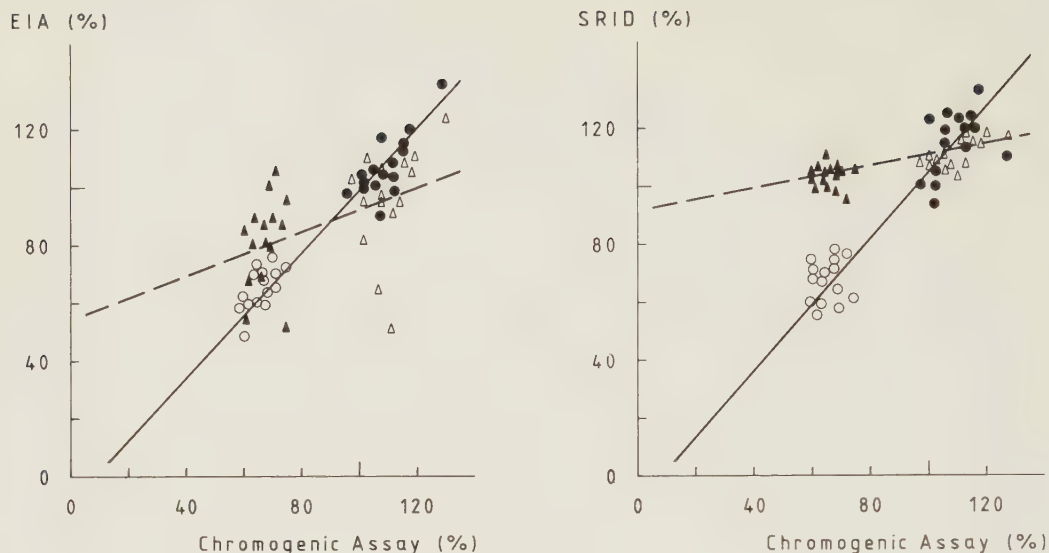


Fig 2. Comparison between antithrombin III concentrations determined by a chromogenic activity assay and with EIA (A) and SRID (B) respectively. For EIA agarose types ME (circles) and HSC (triangles) are compared, and SRID-agarose types B (circles) and HSC (triangles). Plasma samples are denoted by solid circles and open triangles respectively; serum samples are given open circles and solid triangles. Equations for lines of regression and correlation coefficients are provided in Table 1.

Table 1. Summary of correlation coefficients and equations for lines of regression after comparison of activity and immunochemical analysis of antithrombin III.

1) Electroimmunoassay	Agarose	Correlation	Lines of regression
n = 30	B	$r = 0.950$	$Y = 5.181 + 0.945 X$
n = 30	ME	$r = 0.832$	$Y = -10.68 + 1.093 X$
n = 30	HSC	$r = 0.482$	$Y = 57.65 + 0.389 X$
n = 30	HSIF	Not tested	Not tested
2) SRID	Agarose	Correlation	Lines of regression
n = 30	B	$r = 0.881$	$\hat{Y} = -8.572 + 1.185 X$
n = 30	ME	$r = 0.859$	$Y = 5.81 + 0.91 X$
n = 30	HSC	$r = 0.636$	$Y = 91.76 + 0.165 X$
n = 30	HSIF	$r = 0.201$	$Y = 107.83 + 0.047 X$

Area of
immunoprecipitate
(mm²)

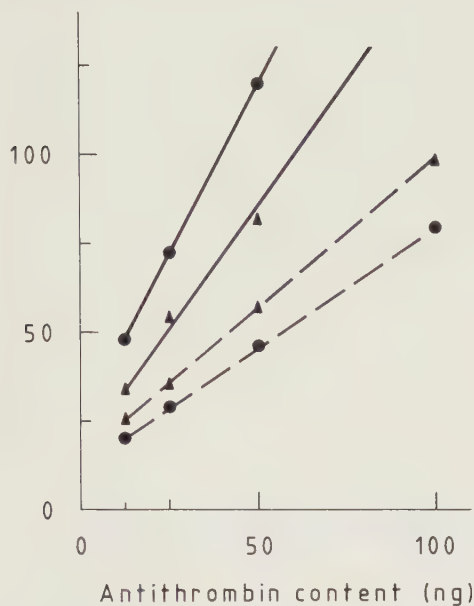


Fig 3. Comparison of pure AT concentration assayed in the presence (solid line) or in the absence (broken line) of heparin. SRID results are represented by triangles and EIA results by circles.

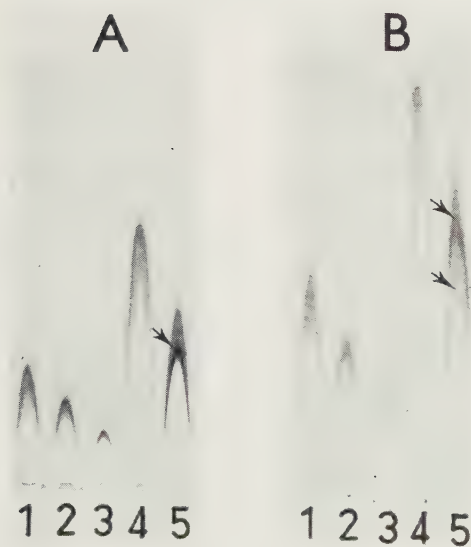


Fig 4. Heparin effect on AT immunoprecipitation during EIA. Results obtained without heparin (A); in the presence of heparin (B). Arrows denote inner precipitin arc identifying AT/protease complexes in serum samples. Antigens: purified AT (1-3), normal plasma (4), and corresponding serum (5).



Fig 5. Electroimmunoassay of AT in plasma (1,3) and serum (1,4) after decreasing the buffer molarity from 75 mmol/L (A) to 25 mmol/L (B) in agarose type ME. Electrophoresis was run at 12 V/cm for 4 hours.

AT/protease complexes was disrupted into several weaker precipitates (Fig 4).

Lowering the ionic concentration of the gel buffer from 75 mmol/L to 25 mmol/L produced decreasing EIA precipitin loop heights in the plasma samples with additional dense precipitin lines inside the loop head (denoted by arrows in Fig 5). The peak heights remained constant, however the inner precipitate became more dense.

DISCUSSION

Plasma and serum contain the same amount of antithrombin III, however, in serum approximately one third is linked to thrombin and thus functionally inactive (5,8). Electroimmunoassay and SRID of AT as investigated in this report provided similar overall results for both plasma and serum (containing significant amounts of AT/protease complexes), however the results varied with the type of agarose used. A high correlation between functional and immunochemically estimated AT (Table 1) was obtained only when sulphated gels were used as the support medium. The precipitin

enclosed area of EIA in desulphated gels was larger and the precipitate margin much less distinct with decreasing sulphate content. This contrasted with the precipitin area of SRID which was considerably smaller in desulphated gels. Clinically acceptable differences in AT values when plasma and serum were analyzed were obtained only in sulphated gels.

The precipitin patterns developed by EIA in serum samples in sulphated gels were characterized by a dense inner line that identified AT/protease complexes (8), and a weaker outer line that represented native AT (Figs 4a and 5a). The heights of the outer precipitin loops were proportional to the sample content of native AT. These distinctive patterns plus the peak heights obtained with EIA indicate that complexed and free AT bind to the same type of antibodies, but the AT/protease complexes link at least one additional antibody directed to an antigenic determinant(s) that emerges during the AT/protease interaction. This additional type of antibodies accelerates and enhances the precipitation of the AT/protease complexes, and may explain why a good correlation between immunoreactive and functional AT assays can be obtained. The plasma-serum difference was diminished when the ionic concentration of the gel buffer for EIA was reduced. This implies a decrease in differences between the solubility of the more soluble antigen-antibody complexes formed with native AT compared to those of AT/protease complexes. Thus low ionic strength buffers should be avoided.

Antithrombin and its protease complexes have affinity for sulphated polysaccharides (13,14). Sulphated agarose gels appear to enhance precipitation of immune complexes of both AT/protease complexes and native AT. These immobilized, charged groups seem to induce anchoring of the AT antigen-antibody complexes to the gel matrix, serving as a precipitation nucleus. Such a concept is supported by our findings that the addition of

heparin to the agarose or to the test samples inhibits immunoprecipitation of AT. Heparin competes with the gel-fixed agarose sulphate groups for the same binding sites on AT. The inclusion of heparin to agarose gels has recently been advocated as a means to reduce the running time required for EIA of AT (15). Our findings indicate that addition of heparin should be omitted, when the prime purpose is to quantitate functionally active AT.

It is evident from these and previous studies (8) that an agarose with medium sulphate content ought to be used for EIA and SRID analysis of AT. The tests should be performed in a buffer with relatively high ionic concentration (e.g. 75 mmol/L) and the antiserum should preferably contain antibodies that recognize and distinguish additional antigenic determinants on AT/protease complexes if the purpose of the immunological analyses is to quantitate functionally active AT.

ACKNOWLEDGEMENTS

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ACTIVATION OF C1,* THE FIRST COMPONENT OF COMPLEMENT, THE GENERATION OF C1r–C1s AND C1 INACTIVATOR COMPLEXES IN NORMAL SERUM, BY HEPARIN-AFFINITY CHROMATOGRAPHY†

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Abstract—Investigations facilitating heparin-affinity chromatography and immunochemical procedures were utilized to study the interaction and behaviour of heparin with C1, C1 subcomponents and C1 IA in normal serum.

Clq bound to heparin-Sepharose independent of divalent cations at 4 and 37°C at physiological pH and salt concentration requiring a high salt concentration for its complete release. Macromolecular C1 bound to heparin-Sepharose in the presence of Ca^{2+} at 37°C, activating C1r and C1s in the process as demonstrated by the appearance of C1r–C1s–C1 IA complexes when purified C1 IA was added to the eluted fractions. At 4°C however, proenzyme C1r and C1s were present in the eluted fractions. Proenzyme C1r–C1s and C1r–C1s–C1 IA complexes did not bind directly to heparin-Sepharose. Binding of C1r–C1s in the presence of Ca^{2+} was dependent on Clq bound to heparin.

High levels of proenzyme C1r–C1s complexes were produced during the 4°C experiments in the presence of Ca^{2+} and were collected in the void volume.

C1r–C1s–C1 IA complexes generated during the 37°C experiments had no affinity for heparin-Sepharose. C1 IA did not bind directly to heparin. Recirculation of a serum sample collected after Clq was removed by heparin-affinity at 4°C in the presence of EDTA demonstrated that (1) C1r and C1s in the presence of calcium could be reassembled with heparin-bound Clq to form macromolecular C1. (2) C1r and C1s in non-activated forms did not bind directly to heparin-Sepharose. (3) C1r and C1s contained in the reassembled C1-heparin complex at 37°C were in active enzymic forms.

INTRODUCTION

The first component of complement in normal human serum is a calcium dependent macromolecular complex composed of Clq, 2 C1r and 2 C1s (Lepow *et al.*, 1963; Gigli *et al.*, 1976; Porter & Reid, 1979). Antigen-antibody aggregates bind Clq, and induce internal activation of the zymogens C1r and C1s to form an active enzymic complex C1, which initiates a sequence of specific protein interactions known collectively as the classical complement pathway (Porter & Reid, 1979). Generated C1 is regulated via C1 inactivator (C1 IA), which binds both active proteases C1r and C1s of the complex thereby controlling further enzymic activity

(Levy & Lepow, 1959; Ratnoff *et al.*, 1969; Harpel & Cooper, 1975).

Heparin has a well known anticomplementary action and induces inactivation of C1 on incubation with normal serum (Rent *et al.*, 1975). Multiple interactions between heparin and the C1 macromolecule have been proposed. It was demonstrated (Loos *et al.*, 1976) that binding of C1 to immune complexes was inhibited by direct interaction of heparin with Clq. The influence of C1s on C4 and C2 could also be prevented by heparin. However there was no evidence for direct heparin interaction with isolated C1s. Rent *et al.* (1976) proposed that heparin enhanced the ability of C1 IA to inhibit the esterolytic activity of C1. Conversely Strunk & Colten (1976) were unable to demonstrate any enhancement of C1–C1 IA complex formation by heparin. Laurell *et al.* (1976) found that the addition of heparin in large enough doses to block C4 conversion in normal serum did not influence the precipitin

*Nomenclature: where applicable the nomenclature agreed by the WHO committee on complement has been used (Bull. W.H.O., 1968).

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patterns of β_1 (Clr-Cls) or α_2 (C $\bar{I}$ r-C $\bar{I}$ s-C $\bar{I}$ IA) complexes.

We have recently reported direct binding and recovery of specified complement proteins following heparin affinity chromatography (McKay & Laurell, 1980). The precise nature by which the Cl macromolecule and heparin interact needs clarification. Therefore a more detailed series of investigations using heparin affinity experiments with fresh normal human serum and the consequent effects on Cl and C $\bar{I}$ IA are reported.

MATERIALS AND METHODS

Fresh serum

This was obtained from an apparently healthy blood donor. The sample was subjected to chromatography within 4 hr of collection, but otherwise stored at -80°C in suitable aliquots until used.

Heparin-Sepharose 4B

This was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden (Batch No. EB 12043).

Cyanogen bromide-activated Sepharose 4B

In this the reactive groups were blocked with 1M ethanolamine and it was used to show that the apparent heparin-Cl interaction was not a direct consequence of non-specific absorption to the Sepharose 4B particles.

Affinity chromatography procedures

All chromatographic experiments were performed essentially as described previously (McKay & Laurell, 1980). A column of heparin-Sepharose was prepared and equilibrated (25 ml wet bed volume), with 0.05 M Tris-HCl, 0.01 M sodium citrate in 0.15 M NaCl adjusted to pH 7.4 to which either 0.02 M CaCl_2 , or 0.02 M Na_2EDTA was added. 25 ml samples were applied directly to the column at a flow rate of approximately 20 ml/hr. Unbound proteins were removed by washing the column with three times its bed volume of the starting buffer. Proteins bound to heparin-Sepharose were eluted using a linear salt gradient consisting of 75 ml 0.1 M Tris-HCl, 0.01 M Na citrate in 0.15 M NaCl, pH 7.4, in the mixing chamber and 75 ml of the same buffer in 1.5 M NaCl in the reservoir. Elution was continually monitored at 280 nm. Affinity experiments were run at 4 and 37°C in the presence of Ca^{2+} or EDTA.

Samples prior to chromatography, the collected void volume concentrated back to the original starting volume, and aliquots from eluted fractions were all subjected to immunochemical analysis.

Immunochemical procedures

Clq, Clr, Cls and C $\bar{I}$ IA present in eluted fractions and void volumes were analysed by electroimmunoassay (EIA) as previously described using monospecific rabbit antisera (Sjöholm, 1975; Sjöholm *et al.*, 1976; Laurell *et al.*, 1979). Anti Clr and Cls reacted with both zymogen and activated forms of Clr and Cls. These proteins when complexed with C $\bar{I}$ IA demonstrated a loss of immunochemical reactivity but could be identified in C $\bar{I}$ IA complexes by using these antisera in combination (Laurell *et al.*, 1978, 1979). Cl subcomponent complexes in selected fractions were further analysed by crossed immunoelec-

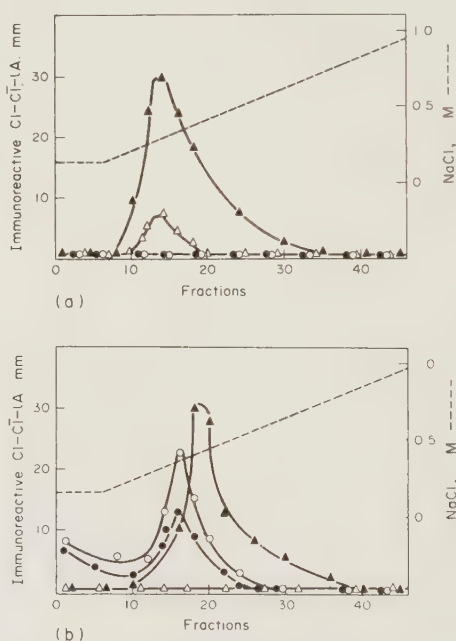


Fig. 1. Elution patterns of Cl subcomponents and C $\bar{I}$ IA following the application of normal human serum to heparin-Sepharose at 4°C and their subsequent release on application of an increasing NaCl concentration gradient. Chromatography was carried out in the presence of 0.02 M Na_2EDTA (Fig. 1a) or in the presence of 0.02 M CaCl_2 (Fig. 1b). Fractions (3 ml) were collected at 4°C . Cl subcomponents and C $\bar{I}$ IA were quantitated immunochemically by EIA and compared directly with the serum sample prior to chromatography. (▲—▲) Clq; (○—○) Clr; (●—●) Cls; (Δ—Δ) C $\bar{I}$ IA; (---) NaCl concentration gradient.

Table 1. Summary of Cl subcomponents and Cl IA which were either bound to heparin-Sepharose in the 4 different affinity experiments or recovered in the void volume

Type of heparin affinity chromatography	Amount of Clq, Clr, Cls and Cl IA bound to heparin					Amount of Clq, Clr, Cls and Cl IA recovered in void				
	Clq	Clr	Cls	Cl IA	Types of complexes*	Clq	Clr	Cls	Cl IA	Types of complexes*
4°C in EDTA	100%	Nil	Nil	5%	Cl IA	Nil	100%	100%	95%	β , α_2
4°C in Ca^{2+}	95%	20%	20%	Nil	γ , β	5%	80%	80%	100%	γ , β , α_2
37°C in EDTA	100%	Nil	Nil	5%	Cl IA	Nil	100%	100%	95%	α_2
37°C in Ca^{2+}	90%	55%	60%	10%	γ , β , α_2	10%	40%	40%	90%	α_2

* Abbreviations used: Cl IA = Cl inactivator complexes of unknown composition, γ = macromolecular Cl, β = Clr-Cls complexes, α_2 = Clr-Cls-Cl IA complexes.

trophoresis as previously reported (Laurell *et al.*, 1978).

RESULTS

Affinity experiments performed at 4°C

Experimental conditions were designed so that all Clq contained in the samples to be chromatographed was bound to heparin-Sepharose at 0.15 M NaCl, in the presence of 0.02 M EDTA. One ml of serum applied to 1 ml of wet gel was found to meet these requirements.

Figure 1a illustrates the elution pattern of Cl subcomponents and Cl IA obtained following the application of normal serum to heparin-Sepharose in the presence of 0.02 M EDTA. All Clr and Cls applied was recovered in the void volume. The bound Clq was released from heparin as a single large peak eluting between 0.2 and 0.65 M NaCl.

Five per cent of total Cl IA applied to the column was retained to be eluted between 0.2 and 0.65 M NaCl (fractions 14–22), the rest was recovered in the void volume (Table 1); the void volume when assessed by crossed immunoelectrophoresis using anti Cls antiserum revealed the presence of β_1 and α_2 precipitin peaks (Fig. 2a). Further analysis with anti Clr and anti Cl IA demonstrated the presence of Clr-Cls complexes in the β_1 and Clr-Cls-Cl IA complexes in the α_2 peak. The Cl IA containing elution fractions (14–22) were concentrated and also analysed with anti Cl IA. A small, diffuse 'filled in' precipitin peak migrating in the α_2 region was demonstrated indicating complex formation. The low concentration of proteins present in these fractions however rendered it impossible to demonstrate immuno-reactive Clr and Cls.

Figure 1b illustrates the elution pattern of Cl subcomponents and Cl IA obtained, following

the application of normal serum to heparin-Sepharose in the presence of 0.02 M Ca^{2+} . Eighty per cent of Clr and Cls applied to the column was recovered in the void together with a very small amount of Clq. Ninety five per cent of Clq applied bound to heparin-Sepharose, the bulk being released on elution as a rather narrow single peak between 0.3 M and 0.5 M NaCl with some trailing to 0.7 M NaCl. Twenty per cent of Clr and Cls applied bound to the column to be released together following a separate elution curve to that of Clq. All Cl IA was recovered in the void volume, none apparently being bound to heparin under the conditions used. The recoveries of Cl subcomponents and Cl IA as determined by electroimmunoassay from the two 4°C chromatography experiments are summarized in Table 1.

The void volume obtained following affinity chromatography in the presence of Ca^{2+} demonstrated three separate Cls containing molecular species—a minor precipitin peak at site of application and large β_1 and α_2 peaks (Fig. 2b). Further analysis using specific anti Cl subcomponent and Cl IA antisera in combination revealed these three peaks to contain Cl (Clqrs), Clr-Cls and Clr-Cls-Cl IA complexes respectively. The high concentration of Cl IA contained in the void separable from Clr and Cls allowed the conclusion that these proteins were in proenzyme form. The initial fractions (1–10) collected immediately prior to application of the elution gradient revealed the presence of Clr-Cls complexes only (Fig. 2c). Fractions in the major elution peak (11–20) contained a mixture of Clr-Cls and macromolecular Cl complexes (Fig. 2d). The addition of purified Cl IA to these fractions did not alter the electromigration of the precipitin peaks indicating that Clr and Cls were in zymogen form.

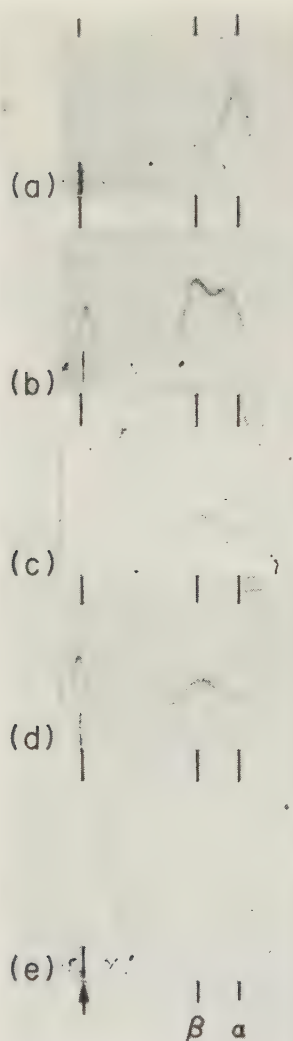


Fig. 2. Crossed immunoelectrophoresis patterns using anti Cls antiserum. Proteins were separated in the first dimension in the presence of 0.02 M Ca^{2+} . The second electrophoretic step was performed in the presence of 0.02 M EDTA with anti Cls in the gel. (a) Void volume following 4°C chromatography with 0.02 M EDTA . (b) Void volume following 4°C chromatography with 0.02 M Ca^{2+} . (c) Initial fraction No. 5 just prior to gradient with 0.02 M Ca^{2+} at 4°C . (d) Elution fraction No. 15 of NaCl gradient with 0.02 M Ca^{2+} at 4°C . (e) Normal human serum examined under same conditions as above. Anode to the right for the separation step and to the top in the second step.

Affinity experiments performed at 37°C

Serum samples prewarmed to 37°C were applied directly to heparin-Sephrose. Heparin-Sephrose was also prewarmed and maintained at 37°C until sample application and column

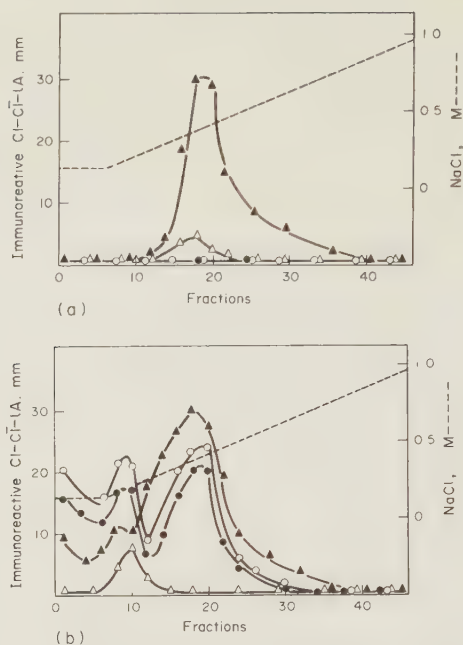


Fig. 3. Elution patterns of Cl subcomponents and Cl IA following the application of normal human serum to heparin-Sephrose at 37°C and their subsequent release on application of an increasing NaCl concentration gradient. Chromatography was carried out in the presence of $0.02\text{ M Na}_2\text{ EDTA}$ (Fig. 3a) or in the presence of 0.02 M CaCl_2 (Fig. 3b). Fractions (3 ml) were collected at 4°C . Cl subcomponents and Cl IA were quantitated by EIA and compared directly with serum sample prior to chromatography. ($\blacktriangle$ — $\blacktriangle$) Clq; ($\circ$ — $\circ$) Clr; ($\bullet$ — $\bullet$) Cls; ($\triangle$ — $\triangle$) Cl IA; (---) NaCl concentration gradient.

washing was complete (approximately 3 hr). All elution steps were carried out at 4°C .

Figure 3a illustrates the elution pattern of Cl subcomponents and Cl IA from heparin-Sephrose in the presence of 0.02 M EDTA following the application of normal serum. Neither Clr nor Cls were bound to the column under these conditions. All Clq applied was bound and subsequently released between 0.2 M and 0.6 M NaCl . Ten per cent of Cl IA was retained on the column and eluted at the same salt concentration as Clq (Table 1), the rest being recovered in the void fraction.

Analysis of this void fraction by crossed immunoelectrophoresis using anti Cls revealed a large α_2 precipitin peak. The Cl IA containing eluted fractions were concentrated. Crossed immunoelectrophoresis using anti Cl IA antiserum revealed a small intensely stained α_2 precipitin peak indicating complex formation; however, immunoreactive Clr and Cls were not demonstrated.

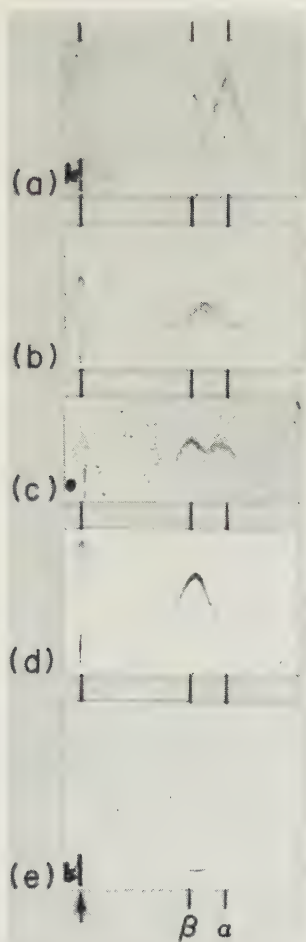


Fig. 4. Crossed immunoelectrophoresis patterns using anti Cls antiserum. Running conditions same as for Fig. 2. (a) Void volume following chromatography at 37°C in presence of Ca^{2+} . (b) Initial fraction No. 1 just prior to salt gradient in presence of Ca^{2+} . (c) Early elution fraction No. 10 of salt gradient in presence of Ca^{2+} . (d) Major elution fraction No. 20 of salt gradient in presence of Ca^{2+} . (e) Normal human serum examined under the same conditions as above. Anodal migration as for Fig. 2.

Figure 3b demonstrates the more complicated elution pattern of Cl subcomponents and Cl IA following affinity chromatography in the presence of 0.02 M Ca^{2+} . Ninety per cent of Clq applied to heparin-Sepharose was bound, while 50–55% of Clr and Cls were retained on the column. Some Clq, Clr and Cls appeared in the initial fractions (fractions 1–5) of the chromatogram prior to the salt gradient. Clq, Clr and Cls followed similar elution curves with minor peaks appearing early in the gradient (fractions 6–11) and the major peaks eluting

between 0.3 and 0.6 M NaCl . Ten per cent of Cl IA applied bound to the column and was released at a similar salt concentration as the early Cl peak.

The void volume revealed a large α_2 Cls precipitin peak by crossed immunoelectrophoresis (Fig. 4a) indicating Clr-Cls-Cl IA complexes (Laurell *et al.*, 1978). The initial fractions (1–5) revealed two Cls reactive peaks—one at the site of application, the other in the β_1 region (Fig. 4b). The fractions eluted early in the salt gradient (6–11) demonstrated three Cls precipitin peaks—at the application site, in the β_1 region and in the α_2 region (Fig. 4c). The fractions from the major elution peak yielded Cls precipitin peaks at the application site and β_1 region only (Fig. 4d). The addition of purified Cl IA to these fractions induced a loss of Cls reactivity from the application site and the appearance of a diffuse precipitate containing immunoreactive Clr, Cls and Cl IA in the slow α_2 region, indicating that Clr and Cls were enzymatically active.

Control chromatography analyses

(a) Chromatographic procedures identical to those above were performed using CnBr-activated Sepharose 4B but without derivatization of heparin. The void volume and eluted fractions were pooled, concentrated and subjected to immunochemical analysis. Less than 10 per cent of Clq was bound to the column with more than 90 per cent collected in the void volume. Clr, Cls and Cl IA were not detected by EIA in the concentrated elution fractions but were fully recovered in the void volume. (b) A heparin-Sepharose column was equilibrated with the starting buffer containing 50 IU/ml of sodium heparin (Vitrum Laboratories, Stockholm, Sweden). Samples prior to chromatography also had heparin added (50 IU/ml). The chromatographic steps followed the identical procedure as above. Concentrated elution fractions contained no detectable Clq, Clr, Cls or Cl IA, all of which were recovered in the void volume, indicating free heparin effectively blocked Cl-heparin-Sepharose interaction.

Dissociation and reassembly of Cl on heparin-Sepharose

This part of the study involved three steps, and is summarized in Fig. 5. (A) Dissociation of Clr and Cls from Clq was attained by applying 25 ml normal serum to heparin-Sepharose at 4°C in the presence of 0.02 M EDTA . Under

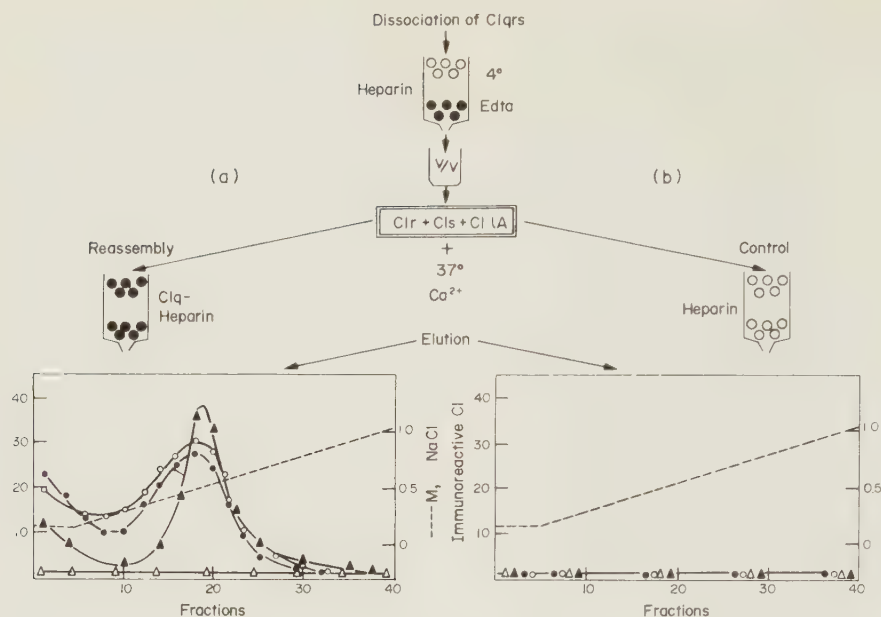


Fig. 5. Dissociation and reassembly of Cl. (a) Macromolecular Cl was dissociated by heparin-affinity chromatography in the presence of 0.02 *M* EDTA (25 ml serum applied). All Clq bound to heparin-Sepharose while Clr, Cls and Cl IA were collected in the void volume. (b) One half of the recalcified void fraction was reapplied to Clq coated heparin-Sepharose at 37 °C in presence of 0.02 *M* Ca²⁺. (c) The second portion of the recalcified void fraction was applied to a new heparin-Sepharose column in the absence of Clq at 37 °C also in presence of 0.02 *M* Ca²⁺. Cl subcomponent and Cl IA eluted from the columns or recovered in the void volume were analysed immunochemically. Clq (▲—▲), Clr (●—●), Cls (○—○) and Cl IA (△—△), (----) NaCl gradient.

these conditions all Clq applied was bound to heparin-Sepharose. The void volume and column washings containing Clr, Cls and Cl IA without Clq, were pooled and concentrated back to the original serum volume. This concentrated fraction was recalcified by dialysis and divided equally. Crossed immunoelectrophoresis of this material (Fig. 6a) using anti Clr, Cls and Cl IA antisera indicated the presence of Clr-Cls and Clr-Cls-Cl IA complexes.

(B) The first portion of the recalcified fraction was reapplied at 37 °C to the Clq coated heparin-Sepharose. Equilibration and affinity chromatography procedures followed as described for previous Ca²⁺ experiments. The void volume was collected and, together with the eluted fractions, analysed by EIA. On application of a linear salt gradient, 95 per cent of Clq on the column was eluted between 0.2 *M* and 0.5 *M* NaCl (Fig. 5). Analysis of this void volume by crossed immunoelectrophoresis with anti Cls yielded a large α_2 precipitin peak (Fig. 6b). Sixty per cent of Clr and Cls applied was bound to the

column and eluted together with Clq. The eluted fractions demonstrated varying ratios of γ and β_1 Cls reactive precipitates indicating the presence of Clqrs complexes. The addition of purified Cl IA to the eluted fractions induced a total loss of Clr antigenic reactivity and a moderate decrease in Cls antigenic reactivity when assessed by EIA. Analysis by crossed immunoelectrophoresis with anti Cls of the same fractions revealed a loss of the precipitin peak in the γ region when Cl IA was added. These results indicated that eluted Clr and Cls were enzymatically active.

(C) The second portion of the recalcified fraction was applied to a fresh heparin-Sepharose column with the same capacity as in (A) using identical experimental conditions as in (B). All Clr, Cls and Cl IA applied were recovered in the void volume. Analysis of this void volume by crossed immunoelectrophoresis demonstrated the presence of Clr-Cls and Clr-Cls-Cl IA complexes in the same ratios as the applied samples (Fig. 6c).

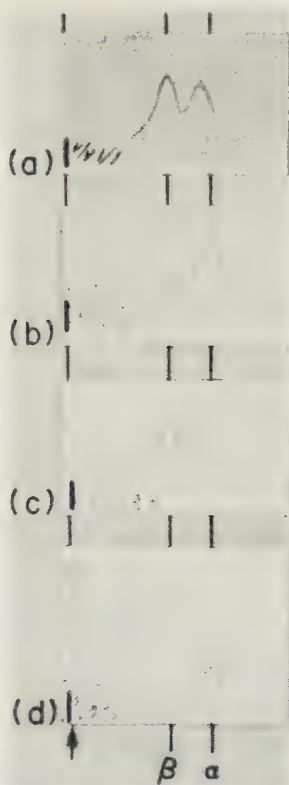


Fig. 6. Crossed immunoelectrophoresis patterns using anti Cls antiserum. Running conditions as for Figs. 2 and 4. (a) Void volume following dissociation of Clq from Cl by heparin-affinity chromatography at 4°C in EDTA. (b) Void volume following recirculation of (a) back onto Clq-heparin complex at 37°C in the presence of Ca^{2+} . (c) Void volume following the passage of (a) over a fresh heparin-Sepharose column at 37°C in the presence of Ca^{2+} . (d) Normal serum run under same conditions as above and provided for reference. Anodal migration as for Fig. 2.

DISCUSSION

Recently we reported a considerable number of proteins in normal serum including Cl subcomponents capable of binding to heparin at physiological salt concentrations (McKay & Laurell 1980). The present study describes heparin affinity studies combined with definitive electro-immunochemical procedures to investigate the direct binding of Cl subcomponents to heparin-Sepharose and the consequent effects. The results provide not only direct evidence for the specific interaction between Clq and heparin described by Raeppe *et al.* (1976) but they also demonstrate that the complex formed did not depend upon divalent cations. Binding

between Clq and heparin, in fact, appeared a little more effective in the presence of EDTA as evidenced by small amounts of Clq recovered in the voided fractions of all affinity experiments containing Ca^{2+} .

Dissociation of Cl with EDTA at both 4 and 37°C induced all Clr and Cls to be recovered in the void volume. This clearly indicates that Clr and Cls in proenzyme form did not bind to heparin directly. Clr and Cls in the presence of Ca^{2+} , however, were retained on the column via Clq in a temperature dependent manner. Twenty per cent of Clr-Cls applied, was retained on Clq bound to heparin-Sepharose at 4°C whereas up to 60 per cent was bound at 37°C. Clqrs in normal serum has been shown previously to be held in dissociation equilibrium (Bartholomew & Esser, 1977). The high levels of Clr-Cls complexes recovered in the 4°C void fraction was probably due to the binding of Clq to heparin with an accumulation of dissociated proenzyme Cls-Cls complexes which have no affinity for heparin. Increased levels of Clr-Cls complexes have been demonstrated in sera of patients with various active disease states (Laurell *et al.*, 1976; Johnson *et al.*, 1977). The findings in the experimental situation described here point to further investigation for the presence of *in vivo* Clq binding substances that may not directly activate Clr and Cls (Prelner *et al.*, 1980).

Clr-Cls-Cl IA complexes in greater concentrations than the applied sample were recovered in the void fractions obtained from affinity experiments in the presence of Ca^{2+} . This indicated that activation of Cl during chromatography took place, followed by some dissociation of Clr-Cls from the Cl macromolecule by Cl IA and is analogous with findings where well known Cl activators have been used (Laurell *et al.*, 1978; Arlaud *et al.*, 1979a; Ziccardi & Cooper, 1979). Furthermore, these results in addition to those obtained from the 4°C Ca^{2+} experiments indicate Clr-Cls-Cl IA complexes *per se* do not bind to heparin-Sepharose.

Virtually all Cl IA (90–100 per cent) applied passed over heparin-Sepharose to be collected in the void fraction in every experiment. This confirms previous observations that Cl IA itself does not interact directly with heparin-Sepharose (McKay & Laurell, 1980), but rather via activated Clr and/or Cls. Rent *et al.* (1976) have proposed, however, that enhancement of Cls-Cl IA complex formation by heparin

depends on the direct action of heparin with C1 IA. Further studies using purified systems are required to elucidate these differences.

In the presence of calcium, C1r and C1s were retained on heparin-Sepharose in the form of macromolecular C1 complexes. At 4°C these complexes contained proenzyme C1r and C1s. The much greater proportion of these proenzymes recovered in the void volume at 4°C (20 per cent only was retained on the column) suggests proenzyme C1r-C1s might be more readily dissociated from C1q and explains in part the different C1r, C1s elution curves obtained from that produced by C1q. Previous ultracentrifugation studies with macromolecular C1 have shown that C1 dissociates at ionic concentrations above physiological levels (Colten *et al.*, 1968). No such difference in C1q contra C1r-C1s maxima was observed in the corresponding 37°C experiments. C1r and C1s contained in these latter elution fractions (Fig. 2b) were shown to be activated which can be explained in part by activation of C1 while C1q binds to heparin, with subsequent disassembly and possible reassociation of active C1r-C1s complex to the column. The early elution peak obtained from the 37°C affinity experiment with Ca^{2+} has not been explained by these experimental procedures and requires further study. The very small amount of C1 IA eluted from heparin-Sepharose in both EDTA and the 37°C Ca^{2+} experiments has not been clarified. Although immunochemical studies indicate this C1 IA to be in complex formation, C1r and C1s were not demonstrated.

The recirculation experiment (Fig. 5) demonstrated that macromolecular C1 can be readily dissociated and reassembled in the presence of heparin. At 37°C, reassembly induced activation of C1r-C1s with considerable amounts of active proteases bound to C1q, even though C1 IA was present during the assembly process. That much activated C1r and C1s remained complexed to heparin-bound C1q may reflect the time lag required for activation of C1r following the reassembly of macromolecular C1 (Dodds *et al.*, 1978). Thus, this relatively slow, rate limiting process may allow most of the C1 IA present in the sample to pass over the assembling C1 before activation has time to occur. Nonactivated C1r and C1s do not bind heparin directly as evidenced from the results obtained and presented in Fig. 5.

It should be emphasized that in the recirculation experiment the amount of C1r-C1s reapplied was less than half that normally contained

in human serum. Therefore, even though reassembly of macromolecular C1 occurred, 40 per cent of the applied C1r-C1s did not bind. Thus, in spite of a relative surplus of C1q bound to heparin reassembly of the macromolecular complex was inefficient. These findings may be explained by several factors. EDTA treatment of serum has been reported to induce decreased C1 functional capacity (Sassano *et al.*, 1972). Whether EDTA *per se* alters the reassembling capacity of C1q or if the structure in C1r participating in C1q binding is adversely affected by EDTA is unknown. Another possibility to be considered is that dissociated C1q may bind to heparin-Sepharose not only by its globular head portion but also—at least partly—by its collagen like tail which may interfere with efficient assembly of C1r-C1s. Many other proteins in normal serum are capable of binding heparin-Sepharose under these conditions (McKay & Laurell, 1980), which could also interfere with optimal assembly of C1. Further studies using C1q purified in the absence of EDTA or closer analysis of heparin binding capacity using enzyme treated C1q may help to elucidate this problem.

Von Zeipel *et al.*, (1977) reported the use of heparin affinity chromatography for purifying C1 from euglobulin. Our studies have demonstrated that C1 under certain conditions may be activated at 37°C in the presence of Ca^{2+} following its binding to heparin-Sepharose. Such a reaction could be similar to the C1 activation process initiated by Ag/Ab complexes (Dodds *et al.*, 1978; Arlaud *et al.*, 1979b).

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BINDING OF PURIFIED C1 SUBCOMPONENTS, C1 INACTIVATOR AND THEIR COMPLEXES
TO IMMOBILIZED HEPARIN

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Key words: C1 subcomponents; heparin; affinity chromatography; C1t(SAP)

SUMMARY

Under specified conditions purified Clq, activated C1r and C1s and C1r-C1s complexes were bound independently of Ca²⁺, to heparin-Sepharose, and could be eluted by an increasing salt gradient. Zymogen C1r and C1s, C1r-C1s complexes, C1 inactivator, and C1r-C1s-C1 inactivator complexes were not bound. However, at lower conductance Ca²⁺ independent binding of C1r occurred, which was utilized in the purification of C1r and C1s. In the presence of C1t (serum amyloid P component), C1s was firmly retained on heparin-Sepharose, which was probably due to formation of a C1s-C1t complex.

INTRODUCTION

The first component of complement, C1, exists as a calcium dependent complex of three subcomponents, C1q and the zymogens C1r and C1s, in a molecular ratio of 1:2:2 (1). Activation of C1 and the classical complement cascade is initiated through the binding of C1q to the Fc portion of IgG or IgM (2). Heparin also binds C1q and inhibits C1 in normal serum at 37°C (3). The activation of C4 and C2 was prevented by heparin without apparent evidence for interaction with C1s (4,5). In addition, heparin potentiated the inhibitory action of C1 inactivator (C1 IA) on C1s (6).

A considerable number of plasma proteins, including C1 subcomponents, are capable of binding to heparin-Sepharose (7). Evidence was produced that zymogen C1r and C1s, C1 IA and complexes containing C1 IA were not bound to heparin-Sepharose (8).

In this report, further studies concerning C1-heparin interactions are documented using purified components. Furthermore, the influence of C1t (serum amyloid P component) on the binding of C1s to heparin was examined.

MATERIALS AND METHODS

Heparin-affinity chromatography. In general, the chromatographic experiments were performed as described previously (7). Unless otherwise stated, affinity columns were equilibrated with Tris-HCl (50 mmol/l), pH 7.4 with NaCl (150 mmol/l) and with Ca^{2+} (20 mmol/l) or EDTA (20 mmol/l), final conductance 20 mS/cm. Proteins bound to heparin-Sepharose were eluted in a linear NaCl gradient up to 1.5 mol/l. After completion of the elution gradient, the column was washed with Tris-HCl (50 mmol/l), pH 7.4, NaCl (1.8 mol/l) and EDTA (20 mmol/l). The eluates were collected in

fractions and tested for the presence of Cl subcomponents.

Immunochemical methods. Eluted fractions, void volume fractions and preparations were analysed for Cl subcomponents and Cl IA by electroimmunoassay and crossed immunoelectrophoresis (9,10,11,12).

Clq was purified as described by Yonemasu & Stroud (13). The preparation contained about 400 $\mu\text{g/ml}$ (300 % of a normal human standard pool).

Preparation of proenzyme Clr and Cls. 400 ml normal human serum was applied to a column containing 350 ml heparin-Sepharose, previously equilibrated with Tris-HCl (50 mmol/l), pH 7.4 containing Ca^{2+} at 2 mmol/l with NaCl added to a final conductance of 15 mS/cm. All chromatographic experiments were performed at 0°C.

About 90 per cent of the total serum protein emerged in the void volume together with about 80 per cent of Clr and Cls. About 95 per cent of the Clq was retained on the column. Clr and Cls bound to Clq on the column, were eluted with the starting buffer now with Ca^{2+} replaced by sodium citrate at 20 mmol/l. Clr and Cls eluted together. Ca^{2+} was added to the pooled fractions at 25 mmol/l.

The pooled and recalcified fraction was applied to 80 ml heparin-Sepharose column, equilibrated with the Tris-HCl buffer (15 mS/cm) containing Ca^{2+} . All Clr and Cls remained on the column. After extensive washing (500 ml), Cls was eluted free of Clr with the Tris-HCl-citrate buffer. Clr remained on the heparin column and was eluted with a linear NaCl gradient ranging from 15 to 45 mS/cm. The pooled fractions were concentrated by ultrafiltration to 300 (Cls) and 500 (Clr) per cent of a normal standard pool.

On agarose electrophoresis in the presence of EDTA the Cls preparation yielded two bands in the α region. The anodal band was identified as Clt

which was present in the preparation at a concentration of 350 per cent of a normal standard pool. No other impurities were detected.

Proenzyme Cls was also isolated by the method of Valet & Cooper (14). This preparation did not contain Clt.

The preparations of Clq, Clr and Cls showed no cross-contamination by immunochemical tests. The Cls preparations did not cleave acetyl-thyrosine-ethyl-ester (ATEe) (15).

Clr-Cl_s complexes were obtained by dilution of 50 ml normal human serum with 400 ml ice-cold acetate buffer (10 mmol/l), pH 5.6, containing 2 mmol/l Ca^{2+} and left for 2 h at 0°C. The precipitate containing macro-molecular Cl was washed with the acetate buffer and dissolved in Tris-HCl buffer (50 mmol/l), pH 7.5, containing NaCl (0.5 mol/l) and Ca^{2+} (20 mmol/l). After centrifugation the dissolved euglobulin was applied to a 10 ml ConA-Sepharose column (Pharmacia Fine Chemicals, Sweden), equilibrated in the same buffer. Clr-Cl_s complexes in zymogen form, free from Clq, appeared in the void volume. After concentration this preparation contained approximately 100 % Clr and Cls present in a normal serum standard pool. A volume of 0.5 ml was applied to a 5 ml heparin-Sepharose column.

C $\bar{\text{I}}\text{r}$ and C $\bar{\text{I}}\text{s}$ were purified as described previously (10,16). The activated state of the C $\bar{\text{I}}\text{r}$ was shown by incubation with purified Cls. The consequent activation of Cls was demonstrated by pH stat titration using ATEe as substrate (15).

C $\bar{\text{I}}\text{r}$ -C $\bar{\text{I}}\text{s}$ complexes were formed by mixing equimolar amounts of purified C $\bar{\text{I}}\text{r}$ and C $\bar{\text{I}}\text{s}$ in the presence of Ca^{2+} .

Cl inactivator (C $\bar{\text{I}}$ IA) was purified as described by Reboul et al (17).

C $\bar{\text{I}}\text{r}$ -C $\bar{\text{I}}\text{s}$ -C $\bar{\text{I}}$ inactivator complexes (C $\bar{\text{I}}\text{r}$ -C $\bar{\text{I}}\text{s}$ -C $\bar{\text{I}}$ IA) were generated in 400 ml pooled normal human serum by incubation at 37°C for 1 h with 1 mg/

ml of heat aggregated IgG (11). The $\text{C}\bar{\text{I}}\text{r}-\text{C}\bar{\text{I}}\text{s}-\text{C}\bar{\text{I}}$ IA complexes were precipitated with the euglobulin fraction obtained by dialysis over night against acetate buffer (20 mmol/l), pH 5.4 containing 2 mmol/l Ca^{2+} . After washing the euglobulin was solubilized in Tris-HCl (50 mmol/l), pH 7.5, containing NaCl (20 mmol/l) and 2 mmol/l Ca^{2+} , and applied to a DE 32 cellulose column (Whatman) equilibrated in the same buffer. $\text{C}\bar{\text{I}}\text{r}-\text{C}\bar{\text{I}}\text{s}-\text{C}\bar{\text{I}}$ IA were eluted by a NaCl gradient and concentrated by ultrafiltration. Further purification was obtained by gel filtration on Sepharose CL 6B (Pharmacia Fine Chemicals, Sweden) as described previously (11). The final product contained $\text{C}\bar{\text{I}}\text{r}-\text{C}\bar{\text{I}}\text{s}-\text{C}\bar{\text{I}}$ IA at about 8 times the concentration of a reference pool of normal human serum treated with heat aggregated IgG (12).

RESULTS

Reactions of purified C1 subcomponents with $\text{C}\bar{\text{I}}$ IA. Zymogen $\text{C}\bar{\text{I}}\text{r}$ and $\text{C}\bar{\text{I}}\text{s}$ preparations did not react with $\text{C}\bar{\text{I}}$ IA whereas $\text{C}\bar{\text{I}}\text{r}$ and $\text{C}\bar{\text{I}}\text{s}$ did (Figs 1, 2). $\text{C}\bar{\text{I}}$ IA changed the electrophoretic position of $\text{C}\bar{\text{I}}\text{r}$ and $\text{C}\bar{\text{I}}\text{s}$ and the precipitates turned diffuse; the heights of the precipitation peaks were decreased when anti $\text{C}\bar{\text{I}}$ IA was combined with anti $\text{C}\bar{\text{I}}\text{r}$ and anti $\text{C}\bar{\text{I}}\text{s}$, respectively (11,18).

On incubation of $\text{C}\bar{\text{I}}$ IA with $\text{C}\bar{\text{I}}\text{r}$ and $\text{C}\bar{\text{I}}\text{s}$ the zymogen states were evident by two non-identifying precipitation lines appearing on analysis with anti $\text{C}\bar{\text{I}}$ IA in combination with anti $\text{C}\bar{\text{I}}\text{r}$ and anti $\text{C}\bar{\text{I}}\text{s}$, respectively.

The addition of $\text{C}\bar{\text{I}}$ IA to $\text{C}\bar{\text{I}}-\text{C}\bar{\text{I}}$ -complexes resulted in the formation of $\text{C}\bar{\text{I}}\text{r}-\text{C}\bar{\text{I}}\text{s}-\text{C}\bar{\text{I}}$ IA as demonstrated by crossed immunoelectrophoresis.

Clq binding capacity of heparin-Sepharose. Saturation experiments using purified Clq demonstrated that one ml heparin-Sepharose was capable of binding about 2.5 mg Clq before the protein appeared in the void

volume. Subsequently heparin-Sepharose columns of 5 ml or more in volume were used to secure excess binding capacity for the purified proteins applied.

Recovery of individual proteins. The amount of the various proteins was assessed with electroimmunoassay of the fractions collected and compared with the amounts applied. Low recoveries were initially obtained particularly with zymogen preparations but on addition of bovine serum albumin (2 %) to the buffers acceptable values were obtained. The low recoveries were apparently due to non-specific adsorption of protein to the collection tubes, which was demonstrated by simple transfer experiments of diluted preparations. The bovine serum albumin did not bind to heparin under the conditions described.

Heparin-affinity experiments with purified components and complexes. Purified Clq, C1r, C1s and C1r-C1s complexes applied separately to the heparin-Sepharose were retained on the column in the presence of Ca^{2+} or EDTA, and were eluted on application of an increasing NaCl gradient. The results are provided in Fig 2.

Under the same experimental conditions zymogen C1r and C1s, C1r-C1s complexes as well as C1 IA and C1r-C1s-C1 IA complexes did not bind to heparin-Sepharose but appeared in the void volume (Fig 3).

The calculated recoveries for the components were between 87 and 103 per cent.

The C1s in the preparation that did not contain C1t appeared in the void volume. In contrast, C1s in the preparation containing C1t, was retained on the column. Part of the C1s was eluted by a NaCl gradient ranging from 20 to 50 mS/cm in fractions that were devoid of C1t. The C1s still remaining on the column at high NaCl concentration was released together with C1t by elution with an EDTA containing buffer (Fig 4).

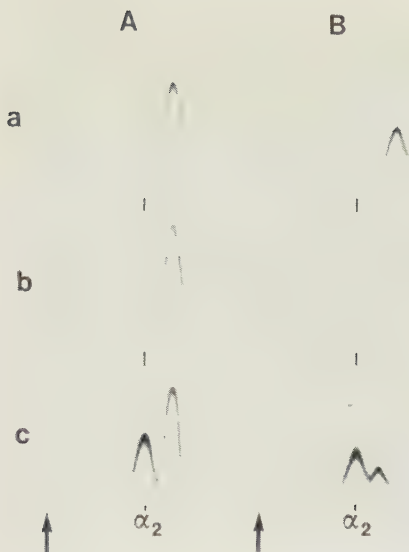
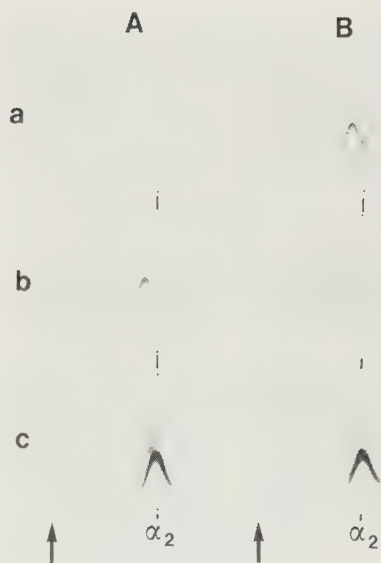


Fig 1. Reaction of Cls and Cls $\bar{}$ with Cl inactivator (Cl IA). Crossed immunoelectrophoresis with both electrophoretic steps performed in the presence of EDTA. Anode to the right. Arrows denote site of application. The α_2 position of normal Cl IA is indicated. A. Zymogen Cls. B. Cls $\bar{}$.
 a. Anti Cls in the gel.
 b. Cl IA incubated with Cls and Cls $\bar{}$, respectively, before electrophoresis. Anti Cls in the gel.
 c. The same as in b but with a mixture of anti Cls and anti Cl IA in the gel.

Fig 2. Reaction of Clr and Clr $\bar{}$ with Cl inactivator (Cl IA). Crossed immunoelectrophoresis with both electrophoretic steps performed in the presence of EDTA. Anode to the right. Arrows denote site of application. The α_2 position of normal Cl IA is indicated. A. Zymogen Clr. B. Clr $\bar{}$.
 a. Anti Clr in the gel.
 b. Cl IA incubated with Clr and Clr $\bar{}$, respectively, before electrophoresis. Anti Clr in the gel.
 c. The same as in b but with a mixture of anti Clr and anti Cl IA in the gel.



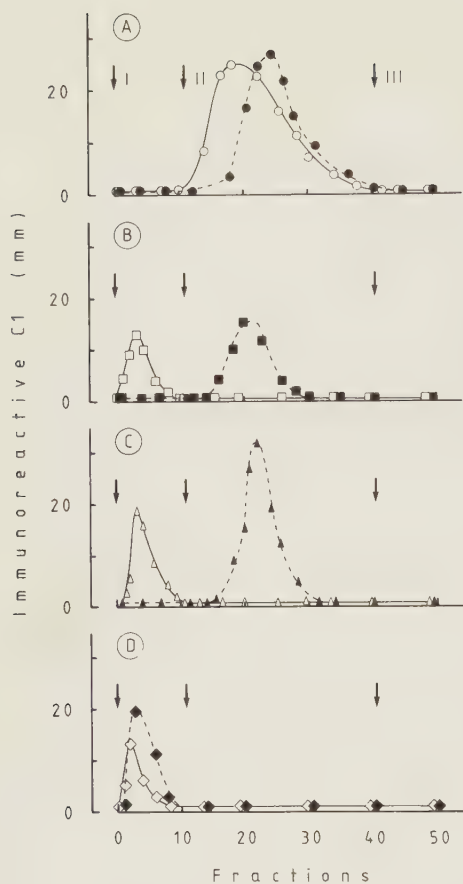


Fig 3. Elution patterns of Cl subcomponents, Cl inactivator and their complexes following application to heparin-Sepharose at 4°C. A. Clq applied to the column in the presence of Ca²⁺ O—O, or EDTA ●—●. B. Clr applied in zymogen form □—□ or activated form ■—■. C. Cls applied in zymogen form △—△ or activated form ▲—▲. D. Cl inactivator ◇—◇ and Clr-Clis-Cl inactivator complexes ◆—◆ applied to heparin-Sepharose.

Arrows indicate I) start of void volume fractions (20 mS/cm), II) start of NaCl gradient (20-150 mS/cm), and III) application of EDTA containing buffer.

The fractions containing Cls, but not Clt (19-23, Fig 4), were pooled and precipitated by ammonium sulphate at 75 per cent saturation. The precipitate was dissolved in distilled water and desalted by passage over Sephadex G 25 (Pharmacia Fine Chemicals, Sweden). This Cls preparation, at a concentration of about 100 per cent of a normal standard pool was reapplied to a heparin-Sepharose column. As shown in Fig 3, all the Cls was recovered in the void volume.

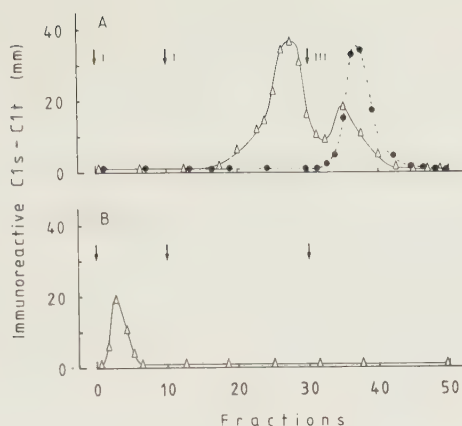


Fig 4. Influence of Clt (serum amyloid P component) on the elution of Cls from heparin-Sepharose.
 A. Elution of Cls (Δ — Δ) and Clt ($\bullet$ — $\bullet$) following application of a mixture of the two proteins in the presence of Ca^{2+} .
 B. Elution of Cls in the absence of Clt (pooled fractions 19-23 from A).
 Arrows indicate I) start of void volume fractions (20 mS/cm), II) start of NaCl gradient (20-150 mS/cm), and III) application of EDTA containing buffer.

DISCUSSION

Complement activation by polyanions has been reported (3,4,5,19,20). Certain sulphated polysaccharides react with proteins of both the classical and the alternative complement pathways (3,7,20,21,22). Heparin binds directly to Clq and also interferes with C4 and C2 activation (3,4,5). Recently we found that C1 could be activated during heparin-affinity chromatography of normal human serum (8).

Experiments utilizing matrix-bound heparin allow isolation and identification of native and activated complement proteins. In the present study, the interaction between heparin and purified C1 subcomponents and C1 IA was investigated. Clq was directly bound to heparin-Sepharose independently of divalent cations and required a high salt concentration

for complete release. The trailing observed during elution of Clq from the affinity column was a consistent finding whether whole serum (8) or purified Clq was used. This may reflect either a heterogeneity of Clq or the existence of different Clq binding sites on heparin.

Using a buffer with a final conductance of 20 mmol/l, zymogen Clr and Cls were previously found to be retained on heparin-Sepharose only in the presence of Clq and Ca^{2+} (8). This was confirmed in the present study with purified Clr and Cls zymogens. However, at a lower conductance of the buffer (15 mS/cm) Clr was bound to heparin-Sepharose independently of Ca^{2+} , a finding that was utilized as a means of purification.

Highly purified Cls prepared according to Valet and Cooper (14) showed no affinity for heparin-Sepharose (Fig 3). However, in the presence of Clt and Ca^{2+} , Cls was retained (Fig 4). In accordance with the findings of Thompson and Enfield (23), Clt was eluted only after addition of EDTA to the NaCl gradient. In our experiments part of Cls was eluted by NaCl, another part appeared together with Clt. The results may imply dissociation of Cls from a Cls-Clt complex. This interpretation was supported by the lack of affinity for heparin-Sepharose in rechromatography experiments with the Cls portion separated from Clt.

In contrast to zymogen Clr and Cls, Clr and Cls bound to heparin-Sepharose independently of Clq and Ca^{2+} at 20 mS/cm. The finding appears to be analogous to those reported for prothrombin which under physiological salt concentrations does not bind to heparin. However, once activated to thrombin, it binds heparin rather firmly (24).

Purified Cl IA and Clr-Cls-Cl IA complexes showed no affinity for heparin-Sepharose which confirms earlier findings using whole serum (8).

Taken together with the observed affinity of Clr and Cls for heparin these results may have a bearing on the reported potentiation of Cls

inhibition by C $\bar{I}$ IA in the presence of heparin (6). Binding of C $\bar{I}$ s to heparin may produce a conformational change of the molecule resulting in increased affinity of the protease for the inhibitor. This possibility is subject to further investigation.

The present findings and earlier reports on interactions of sulphated polysaccharides with the complement suggest a multifaceted regulatory role for heparinoids - a constituent of most cell surfaces (25).

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A SIMPLE TWO-STEP PROCEDURE FOR THE PURIFICATION OF PLASMA Clq
FROM DIFFERENT ANIMAL SPECIES

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SUMMARY

Plasma Clq from human, rat, rabbit, dog and sheep were isolated by a 2-step affinity chromatography procedure. In the first step the method exploits the affinity of Clq for heparin and in the second the interaction between Clq and IgG. The precipitation of Clq by the SO_4^{2-} groups in agarose gels was used as means to rapidly monitor the elution of Clq. This interaction was found to be non-species specific and therefore obviates the need for immunochemical and/or haemolytic assays. The isolation procedure is rapid, simple and is not confined to one species. Pure functionally active Clq was obtained from all species in yields of approximately 85-95 %.

INTRODUCTION

The binding of Clq to the Fc domain of IgG contained in antigen/antibody aggregates initiates a series of sequential protein-protein interactions collectively known as the classical complement pathway (1,2). Clq can however bind directly to IgG that is not in complex with antigen (3), to various polyanionic compounds (4,5) and precipitates at low ionic concentration (1,2,6). Such properties have been exploited as means of isolating Clq from complex biological fluids with widely varied recovery rates and purity (3-6).

In view of the importance complement has as a fundamental biological system in the animal kingdom (2), interest in the biochemistry and structure of Clq has also focused on animals other than man. The unusual amino acid composition of human Clq, its collagenlike tail region and its subunit structure have also been demonstrated in certain other animal species (7,8). In this report a simple, isolation procedure for Clq is described that is suitable for several different species, utilizing properties more or less unique for Clq.

MATERIALS AND METHODS

Samples. Pooled human, rabbit or dog serum, and plasma from sheep or rat were used as starting material.

Reagents. Agarose ME with a sulphate content of 0.15 % (batch No 61639) was supplied from Marine Colloids, Rockland, USA. Heparin-Sepharose 4B, gradient slab PAGE plates (PAA 4/30), CNBr activated Sepharose 4B and DEAE-Sephadex A50 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Antihuman Clq was purchased from Dakopatts, Copenhagen, Denmark. Rabbit anti-serum against whole human and rat sera was available at the laboratory. Monospecific rabbit anti-human Clq,

Clr and Cls were kindly supplied by Dr A-B Laurell.

Whole rabbit serum was first chromatographed over DEAE-Sephadex A50 equilibrated with 0.05 M Tris-HCl pH 7.1. The "break through" IgG containing fractions were pooled and precipitated with ammonium sulphate (40 % saturation) and dialysed extensively against the conjugation buffer (0.1 M NaHCO₃ in 0.5 M NaCl pH 8.1). An IgG affinity column (R-IgG Sepharose) was prepared by coupling rabbit IgG to CNBr activated Sepharose according to the manufacturers' instructions. The coupling efficiency proved to be approximately 10 mg of IgG bound per 1.0 ml of wet gel.

Chromatography Procedures. Heparin-affinity chromatography was performed essentially as described before (9) but in the presence of 0.02 M EDTA. The column was washed with three times its bed volume using the equilibration buffer (0.05 M Tris-HCl, 0.15 M NaCl, 0.02 M EDTA). Proteins bound to heparin-Sepharose were eluted by an increasing linear NaCl gradient. The Clq containing fractions were pooled and dialysed against 0.05 M Tris-HCl, 0.02 M EDTA in 0.1 M NaCl and applied to a column of R-IgG-Sepharose (total IgG bound — 250 mg) equilibrated with the same buffer. Clq was eluted from the IgG column with an increasing linear NaCl gradient.

Clq Detection. Localization of Clq eluted during chromatography was monitored by electroimmunoassay (10) and/or by precipitation of Clq induced by galactose SO₄²⁻ groups contained in agarose gels. The latter occurs during conventional agarose gel electrophoresis at pH 8.6 and can be visualized immediately after electrophoresis without the need of fixation or staining. The length of the precipitation track produced by the migrating Clq during electrophoresis was found to be linear with Clq concentrations between 30 and 500 µg/ml (11).

Characterization of Clq. Agarose gel electrophoresis was performed according to Johansson (12) and SDS/PAGE according to Weber and Osborne (13). The treatment of samples for PAGE and molecular size estimates were performed as described by Yonemasu et al (8) using known marker-proteins (thyroglobulin 669,000; ferritin 440,000; catalase 232,000; LDH 140,000, and bovine serum albumin 67,000). Purified Clq recovered from all species were estimated from their absorbance at 280 nm using $A_{1\text{cm}}^{1\%} = 6.8$ (7). The purity of human and rat Clq was additionally checked by crossed immunoelectrophoresis. All preparations were assessed for precipitating activity with rabbit IgG (4) and for immunological cross-reactivity against anti-human Clq by double immunodiffusion.

The functional activity of isolated Clq preparations was assessed in two ways: a) Whether purified Clq was capable of reforming macro-molecular C1 with C1r-C1s complexes contained in Clq depleted human serum and b) Whether these assembled preparations were haemolytically active. Clq was removed from human serum by the addition of Sepharose 4B conjugated specific anti Clq antibodies (2 mg/ml of gel) in the presence of 0.02 M EDTA and 0.001 M benzamidine. Specific rabbit anti Clq IgG was obtained first by solid phase immunoabsorption. This was used to minimize the level of non anti Clq antibody containing rabbit IgG for conjugation to CNBr Sepharose 4B. Clq depleted serum was recalcified in the presence of benzamidine. Reassembled C1 was assessed by crossed immunoelectrophoresis using monospecific rabbit anti-human C1s antiserum. Haemolytic activity of the reassembled preparations was analysed by a "haemolysis in gel" technique (15). Amboceptor sensitized sheep erythrocytes were added to molten agarose (at 45°C), and allowed to gel, forming a 1 mm thick, homogeneous carpet of indicator cells in agarose. Circular wells (4 mm diameter) were punched from the gel and 10 μ l samples

applied. Normal serum by this method demonstrated decreasing zones of lysis to a dilution of 1:8. Undiluted, Clq depleted serum by contrast failed to produce any lysis. Haemolytic capacity of Clq depleted human serum after addition of Clq preparations were compared to that obtained with normal human serum.

RESULTS

Initial experiments performed demonstrated that all Clq contained in 3.0 ml of human serum could be removed by 1.0 ml of heparin-Sepharose. This assessment was made by comparing the recovery levels of Clq eluted from the heparin column with the levels contained in serum before chromatography by EIA. Each ml of rabbit-IgG Sepharose was capable of removing all Clq from 30 ml pooled human serum also determined quantitatively by EIA. Fig 1 illustrates the slightly varying elution patterns of Clq following chromatography of the different animal sera over a) heparin-Sepharose and b) over R-IgG Sepharose. Fig 2 provides the agarose electrophoretic patterns of the pooled eluted fractions following heparin and IgG affinity chromatography with normal human plasma.

Clq if heated for 30 min at 56°C no longer bound to heparin or IgG. Euglobulin preparations (6) of pooled serum, in our hands, contained a significant portion of Clq (15-30 %) that did not bind to IgG-Sepharose. Presumably this was due to irreversible aggregation of Clq and/or complex formation with IgG that may occur during euglobulin precipitation.

From these results a simplified step-wise elution was designed, i.e. Clq was removed from heparin-Sepharose with the equilibration buffer but now containing 1.8 M NaCl. The Clq rich fractions were pooled, and dialysed against 0.05 M Tris-HCl, 0.02 M EDTA in 0.1 M NaCl before application to R-IgG Sepharose. Elution from the R-IgG column was

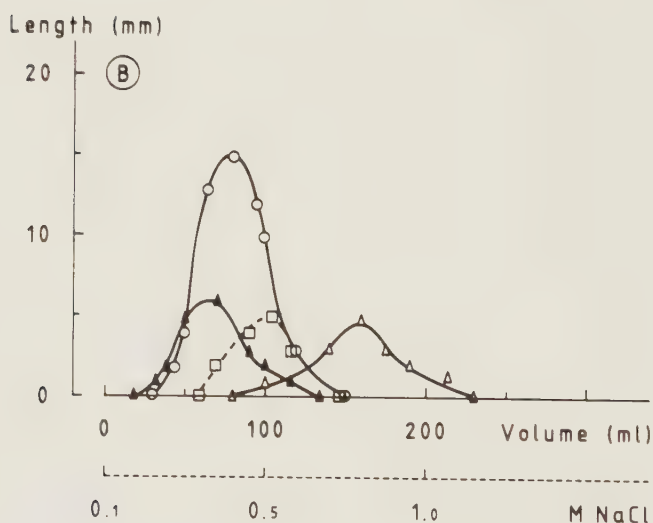
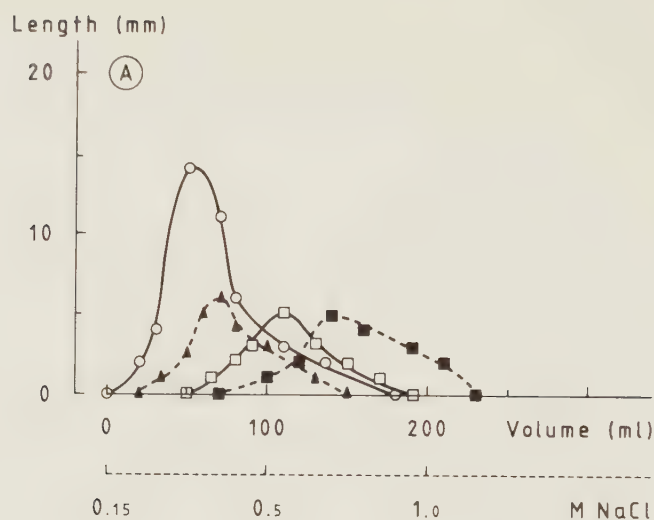


Fig. 1. The elution patterns of different Clq from various species following A) heparin affinity and B) rabbit-IgG affinity chromatography. ○—○ human Clq; △—△ dog Clq; ▲—▲ rabbit Clq; ■—■ sheep Clq and □—□ rat Clq estimated from the Clq/agarose gel precipitation reaction during electrophoresis. Rabbit Clq demonstrated the same heparin affinity as human Clq, and sheep Clq the same R-IgG affinity as human Clq and therefore they are not illustrated.

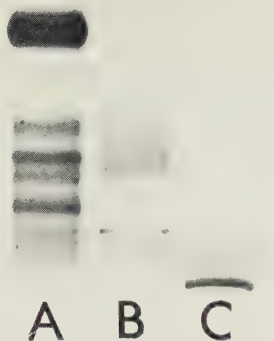


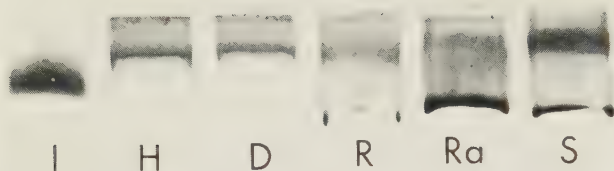
Fig 2. Agarose gel electrophoresis in barbital buffer containing 0.002 M EDTA pH 8.6 20 V/cm for 45 minutes. 10 μ l samples were applied.

- A) Normal human plasma (1:5 dilution).
- B) The pooled Clq rich eluted fractions following heparin affinity chromatography of human serum.
- C) The pooled Clq rich eluted fractions following R-IgG affinity chromatography of B).

achieved with a buffer containing 0.05 M Tris, 10 % Sucrose, 0.05 M EDTA in 1.0 M NaCl pH 8.0 (20). The isolated Clq was stored in this buffer at a concentration of 2-4 mg/ml at 4°C. Table I summarizes and compares the purification steps and recovery of Clq from all species tested.

Isolated Clq invariably produced a single cationic protein band by agarose gel electrophoresis with intense protein trailing from the site of sample application. Clq precipitation in agarose gels for all five species is illustrated in Fig 3 and demonstrates the similarity in results whether the gels were fixed, pressed and stained (3a) or simply pressed and stained without fixation (3b). An IgG 'M' component with cathodal migration was included for comparison. SDS-PAGE analysis revealed single protein bands under non-reducing conditions. By comparison with known marker-proteins, the molecular size of Clq obtained was estimated to be: human Clq 415,000; dog Clq 405,000; rabbit Clq 395,000; sheep Clq 385,000 and rat Clq estimated to be 420,000. All species Clq isolated, revealed non-covalently linked subunits in the presence of urea and multiple covalently linked peptide chains after reduction and alkylation similar to that described for human Clq (2). Purified human and rat Clq demonstrated single cationic precipitin arcs

A



B

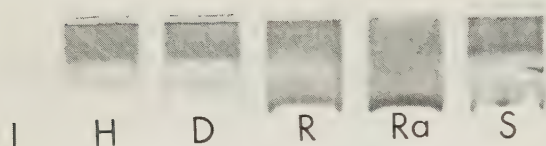


Fig 3. Clq/agarose gel electrophoresis of the purified Clq from different species, run under the same conditions as for Fig 2.

a) The gel was fixed in picric acid, pressed dry and stained in 0.05 % kenacid blue.

b) The gel was pressed without fixation and stained demonstrating the loss of IgG protein only from the gel.

I) human IgG myeloma protein (control); H) human Clq; D) dog Clq; R) rat Clq; Ra) rabbit Clq; S) sheep Clq.

A

B

C

δ β α

Fig 4. Crossed immuno-electrophoresis demonstrating the reassembly of Cl following the addition of purified rat Clq (150 μ g/ml) to human Clq depleted serum using anti Cls antiserum.

A) Normal human serum.
B) Clq depleted normal human serum (A).
C) Rat Clq added to (B).

γ) denotes macromolecular Cl;
 β) Clr-Cl complexes and
 α) Cls-Cl inactivator complexes.

Table I. Purification of Clq.

	Steps	Volume (ml)	Total Protein (mg)	Clq (mg)	Recovery* (%)
A	1 Human Serum ^(a)	600	43500	87	100
	2 Hep-Sepharose	150	675	84	96
	3 R-IgG Sepharose	30	82	82	94
B	1 Rat Plasma ^(b)	150	10700	14	100
	2 Hep-Sepharose	40	152	13	96
	3 R-IgG Sepharose	5	12	12	85
C	1 Rabbit Serum ^(b)	250	18200	28	100
	2 Hep-Sepharose	45	162	25	89
	3 R-IgG Sepharose	15	265	25	89
D	1 Dog Serum ^(b)	250	17200	32	100
	2 Hep-Sepharose	55	87	28	87
	3 R-IgG Sepharose	15	27	27	84
E	1 Sheep Plasma ^(b)	250	14800	35	100
	2 Hep-Sepharose	65	183	32	91
	3 R-IgG Sepharose	15	30	30	85

*These figures are the mean of duplicate runs and are approximate ($\pm 10\%$) as they have been assessed from the values obtained solely by the Clq/agarose gel interaction.

a) Clq measured by EIA and Clq/agarose interaction.

b) Clq measured by Clq/agarose gel interaction only.

when tested by crossed immunoelectrophoresis with rabbit anti-total human and rat serum, respectively.

Clq isolated from all species demonstrated an ability to form macromolecular C1 with C1r-C1s contained in Clq depleted human serums as shown for rat Clq by crossed immunoelectrophoresis (Fig 4). The reassembled C1 preparations were haemolytically active when tested by the "haemolysis in gel" technique.

Antiserum monospecific for human Clq was produced in rabbits within six weeks and did not require absorption. A single precipitin line of complete immunological identity with Clq in human serum and the purified component was obtained by immunoelectrophoresis and immunodiffusion.

This anti Clq produced a reaction of complete identity with known anti Clq antisera from two other sources. Clq preparations from the four other species also demonstrated weak but definite precipitin lines of partial identity with anti-human Clq.

DISCUSSION

This procedure appears to be both specific and gentle for the isolation of Clq as both ligands used apparently bound only functionally active Clq. The utilization of excess ligand over the amount of Clq to be applied in both affinity steps ensures high recovery of Clq from the species tested (85-94 %).

Heparin-affinity chromatography appears to be a more efficient and selective first step in the isolation of Clq than euglobulin precipitation. Clr and Cls are dissociated from Clq in the presence of EDTA and pass out in the void volume (21,22). Clt does not bind heparin in the presence of EDTA (23), and IgG does not bind to heparin at 0.15 M NaCl (11).

An interaction between Clq and agarose has been previously recognized (17,18). However, Assimeh et al (19) considered it to be caused by contaminating IgG while Pohl et al (20) considered it to represent some form of altered Clq. Our results indicate that the Clq/gel interaction is limited to biologically active component as heat treated Clq and the non IgG-binding Clq fraction in euglobulin preparations remained at the site of application without cathodal migration or precipitation.

Monitoring of eluted Clq from the affinity columns by its precipitation during electrophoresis in agarose gels appears to have some advantages over the more commonly used immunochemical and Clq specific haemolytic assays. Although much less sensitive, it is rapid as results can be obtained within 1 hour. This agarose gel interaction appears selective for Clq but not

species specific. Haemolytic assays specific for Clq can apparently be misleading because of the presence of inhibitory substances (16).

SDS-PAGE electrophoresis demonstrated that Clq isolated from all species studied were homogeneous with molecular sizes ranging from 385,000 to 420,000 indicating there was little significant difference between the species.

Cl assembly experiments followed by haemolytic assays indicated that Clq preparations from all five species studied were able complex with human Clr/Cl_s and form haemolytically active Cl.

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